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ABSTRACT BOOK

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Acknowledgements: K.K. was supported by the Project No. 1.1.1.8/1/24/I/003 “Strengthening the Research and Development Capacity of Doctoral Studies at the University of Latvia in the Fields of Smart Specialisation”Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Immune Cell Subsets in Glioblastoma Microenvironment 39

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ORAL ABSTRACTS

Session 1:
Plenary session I

The Frontiers of Cytometry - we are not alone...

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Cytometry is at the very heart of our internationally renowned Imaging and Cytometry Facility at the University of York. We have been very fortunate to have been the first site for many innovations, from the first live-cell population studies using Quantitative Phase Imaging (Marrison et al, 2013 DOI: 10.1038/srep02369), through to beta testing for Carl Zeiss in both light and electron microscopy, and the very first interactions with many the Beckman Coulter CytoFLEX family of cytometers.
As a multi-user core facility, serving a diverse range of sample and application types, beyond the more common applications of blood and monocultured cells, we also research into more niche applications such as microorganisms and plant materials. Dye types are also wonderfully varied, from your classic antibody stains to the latest fluorescent proteins.
The talks will hopefully give a light touch (pun intended) overview of many of these projects, how thet are underpinned by these technologies, and how the CytoFLEX family of cytometers have been instrumental to this work over the past 10 years.

Advances in Molecular Diagnostics in Oncology by Flow Cytometry

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For several decades, flow cytometry–based immunophenotyping has provided critical information for the diagnosis, classification, and monitoring of haematological malignancies, and to a lesser extent, non-haematological cancers. Traditionally, flow cytometry data have been used to determine tumour cell lineage and stage of maturation, enabling accurate classification of leukaemia and lymphoma. In addition, the identification of aberrant immunophenotypic patterns has allowed clear discrimination between normal and malignant cells, both at diagnosis and during follow-up, facilitating sensitive detection of minimal/measurable residual disease (MRD).

In parallel, an increasingly detailed understanding of the mutational landscape of neoplastic cells has further refined cancer diagnosis, classification, risk stratification, and disease monitoring through the application of a growing array of molecular technologies. As a result, the identification of underlying genetic alterations at the single-cell level has become clinically highly relevant, particularly for disease classification and for monitoring both haematological and non-haematological malignancies.

In this presentation, we will review the clinical utility of flow cytometry in several key areas: the assessment of clonality in lymphoid leukaemia and lymphoma, multiple myeloma (MM), and their precursor conditions, including monoclonal B-cell lymphocytosis (MBL), clonal T-cell expansions of uncertain significance (T-CUS), and monoclonal gammopathy of undetermined significance (MGUS); the diagnostic orientation toward specific genetic alterations in acute myeloid leukaemia; the detection of fusion proteins in both chronic and acute leukaemias using immunobead-based assays; and the identification of single-point mutations in cell-derived DNA from patients with clonal mast cell diseases, as well as in cell-free DNA from liquid biopsy samples of lung cancer and acute myeloid leukaemia patients.

In summary, this presentation will review recent advances in molecular oncology diagnostics enabled by flow cytometry, highlighting diverse technological approaches and their application across different cancer types.

Cytometry Across Scales: Single-Cell Meets Spatial Cytomics

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Flow cytometry has transformed life science by enabling rapid, high-dimensional profiling of cellular heterogeneity at scale. Full spectral cytometry now supports simultaneous measurement of more than fifty parameters per cell, strengthening deep phenotyping, rare population detection, and systems-level immune and microbial profiling. Yet conventional flow cytometry is intrinsically limited by dissociation. Cells are removed from their native tissue context, and with that, the spatial information that often determines function, communication, and disease biology is lost.

This gap is increasingly important as translational research shifts from identifying cell types to understanding organised cellular ecosystems within intact microenvironments. Spatial tissue cytomics provides the missing dimension. Highly multiplexed, iterative spatial profiling approaches enable high-parameter proteomics and transcriptomics mapping directly in tissue sections while preserving architecture and single-cell resolution. This allows cellular states to be interpreted alongside neighbourhood structure, microenvironmental gradients, and tissue-specific organisation.

This talk will present cytometry across scales as a defining next step for the field. By integrating full spectral cytometry with spatial tissue cytomics, we can build a coherent continuum from single-cell signatures to tissue-level biology. This cross-scale framework is poised to reshape biomarker discovery and mechanistic insight across immunology, oncology, infection biology, and beyond, accelerating more predictive, context-aware translational discovery.

Session 2:
Plenary session II

Flow cytometry: Yesterday, today - but what about tomorrow?

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The history of flow cytometry over the last 60 years is well known. The developments of Mack Fulwyler, Wolfgang Gohde, & Wallace Coulter were followed by the large number of ISAC members who pioneered the technology and the biological applications. Since 1974 there have been 294,000 publications according to PubMed with the key word "flow cytometry".

Instruments moved from sorters to analyzers, reagent companies emerged. The first commercial monoclonal antibodies were produced by Ortho and published in Science in. Soon, several companies started making commercial Mabs conjugated to key cell markers. BD started in 1981, Coulter Immunology in 1983 and between them and other startups like Pharmingen (1987) introduced Mabs to the world of flow. Today, hundreds of companies make Mabs for dozens of fields including flow cytometry. Other companies focused their attention on the fluorescence reagents – the one most notable would be Molecular Probes. As the field of flow cytometry grew, many other companies started creating dyes that were more efficient at different wavelengths of lasers that were available. An excellent example of this was in 2015 when Dr. Bill Telford published a paper using the 561 nm laser, and now, lots or dyes were created to take advantage of that wavelength.

Similarly, in 2012 Dr. Yong Chen demonstrated his Xitogen instruments using APDs as detectors. Soon after Beckman purchased the company and released the instrument as the CytFlex. From this point on, other companies moved to adopt similar approaches using APDs and today almost every company in the business is using APDs for some or all of their detectors. There are other detectors that have potential such as the new generation of SiPMs driven by the huge increase in demand for lidar technology in self driving cars that will allow single photon technologies to emerge.

In 2004 our group presented the first spectral data on single cells in flow demonstrating that moving from a single intensity, to collecting the spectrum as a parameter and patented by Purdue University. This was licensed by Sony in their SP6800 instrument being the first commercial spectral cytometer. Other companies followed this lead with their own versions of spectral flow cytometry until today virtually every player in the field has a spectral instrument.

So, what does the future hold? What is the expectation for tomorrow in the field of flow cytometery? We know that the range and variety of Mabs is enormous, and not likely to be a limiting factor. Similarly with fluorescent reagents although we are always limited by laser wavelengths. One example is the focus the field has had using 350 nm excitation – the most expensive, and the largest profile laser we use. This will likely disappear in the future and move to 375nm demanding new developments in chemistry. Of course, LEDs may expand together will clever optics that will open up new opportunities. Other new approaches will be identified.

Massively Parallel Cytometry (MPC) to Explore the Phenotypic Diversity of Immune Cells

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Understanding the complexity of the immune system is essential for addressing cancers, infections, and autoimmune diseases. While targeted gene knockout followed by system immunomic analysis remains the standard approach for studying gene function, most common diseases such as diabetes, asthma, or autoimmune disorders arise from intricate interactions between multiple genetic factors and the environment. The risk of developing such diseases is almost always controlled by a combination of genes with individually small effects. Collaborative Cross (CC) mouse lines — derived from the crossbreeding of 8 founder strains (CAST/EiJ, PWK/Ei, WSB/EiJ, C57BL/6J, A/J, 129S1/SvImJ, NOD/LtJ and NZO/HlLtJ) — provide a powerful genetic model for studying these polygenic associations through their exceptional genetic polymorphism traced via SNPs. However, their use in immunology has been limited by the poor/unknown conservation of key epitope targets (e.g., CD45, MHCII, Ly6C) across diverse genetic backgrounds.

To address this challenge, we developed a Massively Parallel Cytometry (MPC) pipeline specifically adapted to CC lines. Spleen samples from 8 CC lines and B6/N controls were multiplexed using a combinatorial barcoding strategy, enzymatically dissociated to preserve surface marker integrity, and depleted of erythrocytes and platelets prior to acquisition. A core immunophenotyping panel enabling the identification of 15 major lymphoid and myeloid populations was combined with the Infinityflow machine learning pipeline (XGBoost-based) to impute the expression of 254 surface markers at single-cell resolution across thousands of cells.

Comparative analysis of marker expression profiles (MFI and percentage of positive cells) across CC lines revealed that SCA-1 is specifically upregulated in CD8 T cells from CC60 and CC80 strains at steady state, a pattern consistent with greater sensitivity to inflammatory mediators such as IL-27, IFN-γ, and type I IFN. Furthermore, functional CD8 T cell subset proportions (Naïve, Central Memory) and integrin β7 expression patterns differed significantly across lines, demonstrating MPC's capacity to detect genotype-driven immunophenotypic variation. Ongoing genetic analyses aim to identify genes in these pathways that differentiate CC lines from one another.

This dataset constitutes a valuable resource for designing high-content immunological panels tailored to CC models, and lays the groundwork for future studies investigating immune response dynamics under inflammatory or infectious challenge conditions, with the goal of uncovering genetic polymorphisms associated with differential immune responses across CC strains.

3D Organoid Cultures to Decipher New Drug Targets: Easy Omics Determination with One-Step Cell Recovery

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Three-dimensional (3D) organoid cultures have emerged as powerful in vitro models that mimic as close as possible the structural complexity of native tissues. They provide a physiologically relevant environment for studying disease mechanisms, cellular interactions, and therapeutic responses. By integrating omics technologies — including genomics, transcriptomics, and proteomics — researchers can uncover new molecular pathways and identify novel drug targets directly within these miniaturized organ-like systems.

A key advancement lies in simplifying the workflow for sample preparation: a one-step cell recovery method enables direct and gentle isolation of viable cells or nucleic acids from 3D culture conditions.

This streamlined process preserves cell integrity, reduces technical variability, and facilitates rapid, reproducible omics analyses. Together, these innovation makes 3D organoid systems a central platform for next-generation robotized drug discovery and precision medicine.

Session 3:
Clinical and Translational Flow Cytometry

Unlocking the Potential of Adoptive Cell Therapy by Targeting Tumor Resistance and T Cell Fitness.

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Introduction Clinical application of CAR T-cell therapies has demonstrated antitumor activity but has also revealed key limitations, including insufficient tumor infiltration, limited persistence and functional exhaustion of transferred lymphocytes. These challenges are particularly evident in solid and heterogeneous tumors and suggest that both tumor-intrinsic resistance mechanisms and suboptimal T-cell fitness contribute to treatment failure.

Methods Guided by these clinical observations, we developed integrated strategies addressing both tumor conditioning and adoptive cell optimization. Tumor-directed approaches focused on modulating autophagy as a preconditioning strategy to increase immunogenicity and susceptibility to immune mediated killing. In parallel, T cells were optimized through complementary epigenetic, metabolic and genetic interventions, including reactivation of autophagy-related pathways and dual targeting. Functional performance and persistence were evaluated in advanced in vitro and in vivo models of immunosuppressive and heterogeneous tumors.

Results Autophagy inhibition in tumor cells increased antigen presentation, promoted immune cell recruitment and enhanced intratumoral accumulation of activated T cells, resulting in improved sensitivity to CAR T cell mediated cytotoxicity and superior tumor control in vivo. In parallel, combined epigenetic and genetic optimization of engineered T cells improved product quality by preserving functional heterogeneity, enhancing metabolic fitness and limiting exhaustion under chronic antigen stimulation in particular when dual targeting was applied. These coordinated effects translated into improved persistence and sustained anti-tumor activity within immunosuppressive tumor microenvironments.

Conclusions Starting from clinical limitations observed with adoptive cell therapies, our work demonstrates that combining tumor preconditioning with T-cell product optimization improves tumor accessibility, cellular fitness,and persistence. This integrated framework provides a rational and translatable strategy to enhance the efficacy and durability of cellular immunotherapies in refractory malignancies..

Macrophage Polarization Shapes Therapy Sensitivity in Acute Myeloid Leukemia

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Introduction. Acute myeloid leukemia (AML) is a malignant hematological disorder with a 5-year survival rate of 30%, declining to 5–10% in elderly patients. Increasing evidence highlights the critical role of the tumor microenvironment (TME), which comprises diverse immune and stromal cell populations that promote immune evasion and therapy resistance. Tumor-associated macrophages frequently adopt an M2-like phenotype, contributing to an immunosuppressive milieu that supports leukemia progression. The aim of the study was to analyze the role of macrophage M2-phenotype in AML therapy response modulation.

Methods. An in vitro AML TME model was established by polarizing donor-derived macrophages into M1 or M2 phenotypes and co-culturing them with AML cell lines THP-1, U937, and MOLM13. Macrophages were isolated from healthy donor peripheral blood mononuclear cells (PBMCs) and cultured in complete RPMI-1640 medium supplemented with GM-CSF (5,000 U/mL) for M1 or M-CSF (50 ng/mL) for M2 polarization. Macrophage phenotypes were characterized by cell surface marker expression (CD14, CD80, CD163, IDO1) using flow cytometry (FC).
The impact of THP-1 and U937 cells on the secretory profiles of healthy donor-derived macrophages was analyzed in trans-well insert cultures by measuring IL-6 and CCL22 levels using ELISA. The effect of venetoclax was evaluated in AML cell line co-cultures with polarized macrophages, and cytotoxicity was assessed using a colorimetric CCK-8 assay.

Bone marrow (BM) and PBMC samples from AML patients were analyzed by FC using a panel of myeloid and monocyte/macrophage markers. Cryopreserved, heparinized PBMC and BM samples were provided by the University of Bologna, Italy. The study was approved by the UL Ethics Committee (approval No. 71-35/59).

Results. The macrophage polarization protocol was validated by the expression of CD80 and CD163 markers. Healthy donor-derived macrophage and AML cell line co-cultures resulted in inhibition of IL-6 secretion. Macrophage polarization modulated the AML cell line sensitivity to venetoclax treatment. The FC panel for assessment of myeloid/macrophage immunophenotypes in AML patient BM and PBMC samples was successfully validated for further studies.

Conclusions. AML tumor microenvironment models indicate a shift towards an M2 macrophage phenotype, highlighting macrophages as potential contributors to therapy resistance in AML.

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Loss of CD56 Expression in NK Cells: Challenges, Interpretation, and Diagnostic Significance in Flow Cytometry

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Introduction. Natural killer (NK) cells are innate lymphocytes derived from a common lymphoid progenitor that also gives rise to T and B cells[2]. NK cells are members of the group 1 innate lymphoid cell family and provide rapid immune responses particularly against virally infected and malignant cells without prior antigen-specific activation[2]. NK cells also secrete cytokines such as IFN-γ and TNF-α, thereby regulating the function of macrophages and dendritic cells and contributing to the coordination of immune responses[2]. NK cells are routinely identified in flow cytometry by the expression of surface markers, most commonly CD16 and CD56, making immunophenotypic alterations diagnostically significant[3].

Methods. In the Clinical Laboratory of the Children's Clinical University Hospital in Riga, Latvia, NK cell immunophenotyping is performed using a 10-color *Beckman Coulter Navios EX* flow cytometer. NK cells are routinely determined as part of lymphocyte subset analysis in peripheral blood samples by adding whole blood to a lyophilized monoclonal antibody cocktail. Immunophenotypic data of lymphocyte populations are analyzed using *Infinicyt* software.

Results. Based on CD56 and CD16 expression patterns, NK cells are categorized into $CD56^{bright}/CD16^{neg}$, $CD56^{dim}/CD16^{pos}$, and $CD16^{pos}/CD56^{neg}$ immunophenotypes, with the latter representing a non-classical but diagnostically relevant NK cell population requiring careful immunophenotypic interpretation.

Conclusions. Loss of CD56 expression in NK cells represents an important diagnostic challenge in flow cytometry, as $CD16^{pos}/CD56^{neg}$ NK cells do not correspond to a classical NK cell subset but may represent altered immunophenotypic and functional features[4]. Recognition of this population is essential for accurate immunophenotypic interpretation and for avoiding misclassification during lymphocyte subset analysis. Careful evaluation of $CD56^{neg}$ NK cells may therefore provide clinically relevant information, particularly in the context of immune dysregulation and disease-associated immune alterations[4].

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Mastering Multicolor Flow Cytometry: From Panel Design to Reliable Results

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Multicolor flow cytometry is a powerful tool for in-depth cellular analysis, enabling the simultaneous measurement of multiple parameters within complex biological samples. As panel complexity increases, careful experimental design and optimization become essential to ensure reliable and reproducible results. This educational session will address key principles of multicolor experiment design applicable to both conventional and spectral flow cytometry platforms.

Sex-Based Differences in Immunophenotype in Healthy Blood Donors

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Introduction. Immune reactions to a wide range of antigens—including harmless environmental antigens, self-antigens, tumor-associated antigens, as well as microbial and vaccine-derived antigens—exhibit marked differences between males and females. Growing interest in the biological basis of sex-related immune dimorphism has led to extensive research, with accumulating evidence indicating that sex hormones play a key role in shaping immune cell function. Therefore, we examined potential differences in basic immune phenotypes between males and females.

Material. Peripheral blood from 61 healthy blood donors (31 women and 30 men) was analyzed. Methods: Immune cell immunophenotyping was performed using standard IVD MULTITEST BD antibodies, and samples were analyzed by flow cytometry.

Results. The female group showed a higher percentage of lymphocytes (CD45++), T helper lymphocytes with a higher absolute count, as well as CD4/CD8 ratio, marginal zone-like B cells, class-switched B cells, and CD21low B cells than the male group. The male group was found to have elevated percentages of naïve B lymphocytes, transitional B cells, and plasmablasts. A weak positive correlation with age was found among double positive T lymphocytes, natural killer T cells (NKT) lymphocytes, and CD21low B cells. A negative correlation with age for double negative T lymphocytes, marginal zone-like B cells, and plasmablasts was noted.

Conclusions. The results indicated the importance of creating distinct reference ranges regarding sex and age concerning immunophenotype. Sex and age markedly influence immune cell composition in healthy individuals. Women exhibit higher T helper cell and memory B cell subsets, while men show elevated naïve and transitional B cells and plasmablasts. These differences highlight the need for sex- and age-specific reference ranges and suggest that therapies and interventions targeting the immune system—including immunotherapies, vaccines, and treatments for infections or autoimmune diseases—should consider these variations to optimize efficacy and safety.

Session 4:
Flow Cytometry in Basic and Applied Science

Calibrating the Invisible: Challenges in Standardization for Nanoparticle Flow Cytometry

Kamil Szeliski

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Nanoscale flow cytometry has emerged as a rapidly advancing field with broad applications, including the analysis of extracellular vesicles, virus particles, lipid nanoparticles, and micro- and nanoplastics. New generation cytometers, purpose-built for these applications, have extended the limits of detection to particles as small as 40 nm, enabling unprecedented sensitivity. However, the increased capabilities of these instruments have not yet been matched by equivalent advances in reagents and reference materials necessary for rigorous controls and calibration, and the variety of available reagents can make entry into the field confusing and challenging.

Despite technological progress, a significant number of recent studies still lack proper calibration, standardization, and adequate reporting of controls. This raises concerns about the reproducibility and comparability of results, and in clinical or diagnostic contexts, can potentially lead to misinterpretation with serious consequences. Properly designed calibration strategies, standardization practices, and quality controls are therefore essential for reliable data generation and interpretation.

Current approaches in nanoparticle flow cytometry reveal both opportunities and limitations. Common pitfalls in calibration and standardization are evident, and examples from extracellular vesicle and nanoparticle research highlight these challenges. These examples also illustrate practical strategies to improve reproducibility and quality control, guiding reliable experimental design and data interpretation.

The current landscape of nanoscale flow cytometry is reviewed, highlighting practical insights into implementing standardized workflows and strategies for reliable experimental design and data interpretation. Emphasizing rigorous calibration and control, this talk illustrates approaches that enhance reproducibility, interpretability, and impact.

Identification of Extracellular Vesicle Signatures of Daratumumab Treated Multiple Myeloma

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Extracellular vesicles (EVs) represent promising non-invasive biomarkers that may aid in the diagnosis and risk-stratification of multiple myeloma (MM). Daratumumab (DARA) is a CD38 antibody approved for treatment of MM, and despite the anti-tumour effects of DARA, the majority of patients eventually relapse. Our recently published data reveals that EVs from peripheral blood plasma (PB) and bone marrow aspirates (BM) from patients treated with DARA contain EV markers as well as MM-associated CD38, CD55 and CD59, and PD-L1. CD55, CD59 and CD147 were elevated in MM PB EVs relative to healthy PB EVs. In addition, EV PD-L1 levels are associated with patient response to DARA (Brennan et al. 2022, PMID: 36359760). The aim of this study was to expand the PB EV biomarker panel for improved predictive power as a non-invasive liquid biopsy for monitoring patient response to Daratumumab.

Material. 61 DARA treated MM patients and 12 heathy donors participated in the study. Participation was voluntarily, and written informed consent was obtained from all subjects. EV isolation was performed on platelet free PB and BM samples by differential centrifugation and density gradient ultracentrifugation, and were analysed by flow cytometry and mass spectrometry.

Results. Mass spectrometry analysis revealed that several adhesion proteins and immune cell markers are elevated in MM PB EVs relative to healthy PB EVs, and are associated with patient response to Daratumumab. The expression of 8 of these markers were assessed in all PB and BM EVs by flow cytometry and correlated with the clinical data available. These markers were combined with the proteins from our previous study to create 2 biomarker panels; a MM and DARA response panels. The MM panel differentiates between cancer patients and healthy individuals, achieving an AUC of 0.92 (Sens: 0. 86/Spec: 0.92), while DARA response panel differentiates between long-term DARA responders (2+ years clinical follow-up) from patients progressing on DARA, achieving an AUC of 0.733 (Sens: 0. 80/Spec: 0.91).

Conclusions. The MM and DARA response panels may have potential as a non-invasive liquid biopsy to complement or replace invasive bone marrow sampling for monitoring patient response to DARA.

Practical Aspects and Results of Extracellular Vesicle Analysis by Flow Cytometry

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Introduction: Extracellular vesicles (EVs) are nanoscale membrane structures released by most cell types and are considered promising biomarkers for diagnostics and disease monitoring. However, EV research is technically challenging due to their heterogeneity in size and cellular origin, co-isolation with non-vesicular particles, and the detection limits of standard flow cytometers. There is also a strong need for standardized EV measurement protocols across instruments to enable quantitative comparison of results between laboratories. This study aimed to develop and optimize a practical flow cytometry protocol for EV detection, characterization, and quantification according to MISEV standards, applicable in a core facility environment.

Methods: EVs were isolated by differential ultracentrifugation from both human serum samples and conditioned media of the HCT116 cell line. Measurements were performed using calibrated Cytek Aurora and Beckman Coulter CytoFLEX LX flow cytometers. Size calibration was conducted using NIST-traceable beads and the FCMPass software. EVs were labeled with fluorescent antibodies targeting canonical EV markers (CD9, CD63, CD8) and the membrane dye. Staining conditions, including antibody concentration, dye dilution, and incubation time, were systematically optimized

Results: The optimized staining protocol enabled clear and specific detection of EVs across instruments. Antibody labeling provided reproducible fluorescence signals with minimal background, while specific membrane dye effectively distinguished vesicular from non-vesicular events. Calibration ensured consistent gating of EVs populations and facilitated cross-instrument comparability. The protocol demonstrated robustness for both serum-derived and cell culture–derived EVs, with reliable discrimination of CD9+, CD63+, and CD81+ subpopulations.

Conclusions: The developed protocol allows reliable identification and analysis of extracellular vesicles by flow cytometry following MISEV guidelines. The combination of optimized antibody staining, validated membrane dye, and rigorous calibration supports reproducible EV measurements across instruments. This workflow provides a practical framework for flow cytometry core facilities and users aiming to implement standardized EV analysis.

The use of imaging flow cytometry to evaluate the mechanism of netosis in blood and pulmonary neutrophils in horses.

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Introduction: NETosis is a specialized neutrophil response mechanism that leads to the release of extracellular NETs (neutrophil extracellular traps), composed of chromatin fibers and granule proteins. This process is an important component of the innate immune response, but its excessive or chronic activation promotes organ damage observed, among others, in lung diseases in humans and horses. There are several types of netosis, the basic ones being suicidal (involving chromatin decondensation and membrane disruption) and vital (occurring without cell lysis, with the neutrophil retaining its effector functions). Among the many methods of analyzing netosis in cells, imaging flow cytometry stands out.

Objective: Since netosis plays an important role in the course of equine asthma, the aim of this study was to investigate its activity in peripheral blood neutrophils and bronchoalveolar lavage fluid (BALF) using morphometric methods and extracellular DNA assessment.

Methods: Blood and BALF were collected from six asthmatic horses as part of the diagnostic procedure. The obtained cells were incubated in the presence of 20 nM Phorbol 12-myristate 13-acetate (PMA) and 5 uM A23187 for 3.5 hours at 37°C with the addition of 5% CO2. Unstimulated samples served as controls. Neutrophils were labeled using anti-DH24A antibody. The cells were then dyed with wheat germ agglutinin (WGA), Hoechst, and anti-MPO antibody. NETosis analysis was done using an Amnis ImageStream MKII imaging flow cytometer. Also, extracellular DNA, as a representation of NET formation, was measured using cell impermeable DNA binding dye (SYTOX Green).

Results and discussion: Based on the method of Zhao et al. 2015, a method for morphometric analysis of horse cells was developed, showing both characteristics of cells in suicidal and vital NETosis.

Additionally, since the cytometric analysis only showed cells before their disintegration, complementing the analysis with an assessment of extracellular DNA in the supernatant allowed for the collection of complete data. The studies confirmed the significant contribution of cells capable of netosis in the course of asthma in horses.

Conclusion: The use of flow cytometry with imaging is a modern method that allows for the assessment of changes in cell morphology and their quantitative analysis.

The impact of TLR7 pathway in murine thymic cell development

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The thymic microenvironment is characterized by constitutive, low-level sterile inflammation, including the steady production of Toll-like receptor (TLR)-induced cytokines. This tonic inflammatory signaling is thought to partially arise from endogenous ligands released from dying cells. While inflammation in the thymus is thought to be an integral component of thymic homeostasis, it is still poorly understood how innate inflammatory signaling pathways shape discrete thymocyte maturation stages and non-lymphoid thymic compartments.

In this study we investigate the impact of TLR7 signaling on distinct thymocyte subsets and thymic cell populations in C57BL/6 and AIRE-KO mice. Using both ex vivo fetal thymus organ culture and in vivo intrathymic delivery approaches, we modulate the inflammation in embryonic E16.5 and postnatal (3-4 week old) mouse thymi. After 4-5 days of stimulation with TLR7 ligands, functional changes are analyzed using 4-laser spectral flow cytometer with two 20-color panels, enabling us to analyse over 60 cell types from the lymphocyte, myeloid and stromal cell compartments.

Alterations were most evident within CD4 single-positive (SP) thymocyte compartments, where the augmentation of TLR7 pathway caused a shift from semi-mature towards more advanced maturation stages in both fetal and young thymi of C57BL/6 and AIRE-KO mice. In contrast, accelerated maturation of CD8SP thymocytes was detected selectively during C57BL/6 embryonic thymopoiesis. In addition, increasing inflammation through TLR-dependent signaling significantly reduced CD4-CD8- γδ T cells and increased DN thymocytes, a similar trend was observed in young thymi, suggesting bias toward conventional T cell differentiation. Beyond thymocytes, enhanced innate inflammatory signaling also reshaped the mTEC compartment in C57BL/6 mice, with selective expansion of mTEC I and III subsets and reduction in mTEC IV and mTEChi populations.

Overall our work identifies TLR signaling as an active regulator of thymocyte maturation timing, lineage bias, and stromal composition in the thymus. Manipulating the tonic inflammation in the thymus can open up new potential targets for modulating thymocyte development and thus the adaptive immune response.

Session 5:
Beginners practical session: Flow for Newbies

A walk in a flow Cytometry

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Over time, flow cytometry (FC) has become a highly useful and widely adopted technique across numerous areas of biological and medical research. Despite its broad application, newcomers to the field often encounter significant difficulties when first engaging with this technology. Conducting a self-sufficient flow cytometry experiment requires extensive knowledge spanning multiple disciplines, including optical physics, medicine and/or cell biology, and mathematics, in order to perform all experimental steps correctly and to interpret the results accurately. Acquiring such interdisciplinary expertise is particularly challenging for students trained primarily in biology. Therefore, providing beginners with clear, accessible educational materials that explain the fundamental principles of flow cytometry could substantially lower the entry barrier to the field, promote wider adoption of the technique, attract new researchers and thereby support its continued development.

Flow cytometry is fundamentally based on light, and in particular on fluorescence. When designing a flow cytometry experiment, the researcher must integrate detailed biological knowledge of the sample with a thorough understanding of the fluorochromes being used. Although the sample is not directly observed with the naked eye, the researcher should be able to visualize how the stained biological material would appear under a fluorescence microscope. This conceptual, mental image of the sample should consistently guide the interpretation of histograms and dot plots generated by the flow cytometer.

Compensation is often the moment when flow cytometry stops being intuitive—and quietly demands a bit more respect.

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Introduction: Multicolor flow cytometry combines fluorochromes whose emission spectra overlap and are collected through partially overlapping detector/filter bandpasses. This inevitably produces fluorescence spillover (spectral overlap), where signal from one fluorochrome contributes to measurements in additional channels. If uncorrected, spillover creates a predictable, systematic bias in measured intensities that can distort population placement and compromise interpretation. Compensation is the standard mathematical correction used to account for this optical crosstalk and is therefore fundamental to reliable multicolor experiments.

Methods: This talk uses a practical, beginner-friendly multicolor panel as a working model to explain the origin and consequences of spillover. Excitation/emission spectra and detector bandpasses are introduced to show why spillover occurs and how it propagates across multiple channels. Raw, uncompensated data are used to visualize spillover-driven shifts in population position and changes in signal distribution. Compensation is presented as a linear, matrix-based correction framework that estimates and subtracts spillover contributions using appropriate single-color controls acquired with consistent instrument settings. Emphasis is placed on how robust controls, stable acquisition settings (e.g., PMT/gain), and informed fluorochrome selection reduce error in spillover estimates and limit downstream propagation of technical bias.

Results: Uncompensated multiparametric examples illustrate spillover-related technical artefacts as apparent changes in population position across channels: fluorescence from one marker contributes to other detectors and alters the apparent intensity of unrelated markers. Applying compensation reduces these artefacts and improves marker interpretability by removing measurement bias from optical crosstalk. At the same time, compensation can increase measurement spread in affected dimensions and may yield negative values, reflecting the mathematics of the correction rather than new biology.

Conclusions: Spillover is an unavoidable consequence of fluorescence physics and instrument optics, but its impact can be managed effectively. Understanding compensation as a tool for controlling measurement bias—rather than a cosmetic adjustment—supports better panel design, more robust gating, and more reliable biological conclusions. A solid grasp of basic compensation principles is essential for anyone performing multicolor flow cytometry.

Flow Cytometry and Solid Organ Transplantation: A Perfect Match?

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The utility of flow cytometry in fields ranging from basic cell biology to clinical haematology relies heavily on the quality of the input sample. As researchers attempt to analyse increasingly complex tissue matrices, the demand for standardized and optimized protocols becomes paramount. This presentation provides a rigorous examination of the complete flow cytometry pipeline, focusing on technical precision at every stage. We will examine the specific requirements for processing distinct tissue types to maximize single-cell yield and viability. Detailed attention will be given to the chemistry and timing of staining protocols, specifically addressing the differences between surface phenotyping and intracellular cytokine or chemokine detection. Finally, we will explore strategies for effective cell sorting alongside the fundamental principles required for logical data analysis. This session serves as a vital primer for early-career scientists, ensuring they understand not just the "how," but the "why" behind every step of sample preparation and acquisition.

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Practical presentation: “Sleeves up and hands on!”

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The objective of this hands-on session is to equip participants with the skills and confidence to operate a cytometer independently, covering essential tasks such as setting voltages/gains, adjusting thresholds, and applying appropriate controls. By mastering these practical steps, participants will realize that the principles learned on one system provide a robust foundation for working with any cytometer.

Using a Beckman Coulter CytoFLEX analyzer, we will run a variety of quality control beads commonly found in flow cytometry labs (CytoFLEX Daily QC, Fluorospheres, Rainbow beads, BD CS&T, and nanoVIS) to demonstrate their unique characteristics and capabilities. The session will also include a demonstration on mechanical tissue dissociation to isolate cells, emphasizing the importance of characterizing sample autofluorescence. Additionally, we will analyze microbiological samples to highlight the necessity of viability markers, concluding with a comprehensive overview of the principles of labeling and compensation

Session 6:
Clinical and Translational Flow Cytometry

Desmoglein-2 as a Gatekeeper of Phenotypical Plasticity in Breast Cancer

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Introduction: Maintenance of epithelial integrity represents a critical barrier against cancer cell dissemination, with desmosomes serving as key mediators of intercellular cohesion. Although desmogleins are essential structural components of desmosomes, their regulation and functional role in cancer progression and metastasis remain incompletely understood. Trop-2 (TACSTD2) is an epithelial surface protein with context-dependent roles in tumor biology; however, its contribution to epithelial integrity and metastatic suppression has remained unclear. This study investigates the functional interplay between Trop-2 and desmogleins, with a particular focus on desmoglein-2 (DSG2), in breast cancer.
Methods: CRISPR/Cas9-mediated knockout models of Trop-2 and DSG2 were generated in human and murine breast cancer cell lines. Epithelial integrity and intercellular adhesion were assessed using transepithelial electrical resistance (TEER), permeability assays, and dispase-based dissociation assays. Protein–protein interactions were identified using proximity-dependent biotinylation (miniTurboID) and validated by immunofluorescence, proximity ligation assays, and transmission electron microscopy. Metastatic behavior was evaluated using orthotopic xenograft models, tail-vein injection assays, and chick chorioallantoic membrane assays. Transcriptomic and proteomic profiling of tumors was performed to identify downstream mediators, followed by pharmacological inhibition for functional validation.
Results: We demonstrate that Trop-2 physically interacts with desmogleins, particularly DSG2, and that loss of Trop-2 disrupts DSG2 membrane localization and desmosomal architecture. Deletion of either Trop-2 or DSG2 compromised epithelial integrity, reduced intercellular adhesion, and markedly enhanced spontaneous metastatic dissemination in vivo, despite variable effects on primary tumor growth. Mechanistically, disruption of the Trop-2–DSG2 axis resulted in upregulation of matrix metalloproteinase-14 (MMP14), promoting extracellular matrix remodeling and invasive behavior. Pharmacological inhibition of MMP activity significantly reduced metastatic burden in DSG2-deficient tumors. Analysis of patient datasets further supported the prognostic relevance of combined Trop-2, DSG2, MMP14, and CLDN1 expression patterns.
Conclusions: Our findings identify the Trop-2–DSG2 axis as a critical regulator of desmosomal integrity and epithelial cohesion that suppresses breast cancer metastasis. Disruption of this axis destabilizes desmosomes, promotes MMP14-dependent invasion, and enhances metastatic dissemination, revealing a mechanistically defined vulnerability that may be therapeutically exploitable to limit metastatic progression.
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Decoding Platelet Identity via Flow Cytometric Immunophenotyping

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Thrombocytopathies comprise a diverse group of inherited and acquired platelet disorders characterized by impaired platelet function and heterogeneous clinical presentation. Despite advances in laboratory diagnostics and molecular genetics, a major unresolved challenge in the field remains the insufficient phenotypic definition of many platelet function disorders. In numerous cases, patients present with bleeding symptoms that cannot be clearly assigned to a distinct functional phenotype, resulting in inconclusive laboratory findings and diagnostic uncertainty.

Platelet function is a multistep process involving adhesion, activation, secretion, aggregation, and procoagulant activity, all of which are dynamically regulated and may be selectively affected in thrombocytopathies. Conventional assays often assess these processes in isolation or provide global functional readouts, thereby failing to capture the full functional spectrum of platelet behavior. As a result, subtle, pathway-specific, or stage-restricted abnormalities frequently remain undetected.

Comprehensive platelet phenotyping therefore requires the ability to evaluate multiple functional stages simultaneously and at the single-platelet level. Flow cytometry provides a unique platform for such integrated analysis, enabling one-time, multiparametric assessment of platelet identity that reflects receptor expression, activation signaling, secretion capacity, and procoagulant transformation within the same sample. By combining constitutive platelet markers with activation-dependent readouts, flow cytometry enables platelet function to be interpreted along a spectrum of phenotypic responses rather than as a simple normal or abnormal outcome.

We focus on how flow cytometric phenotyping can help bridge the phenotypic gap in thrombocytopathies. Emphasis is placed on platelet identity as a spectrum of functional states rather than a single measurable endpoint. Through detailed evaluation of platelet responses at the single-platelet level, flow cytometry supports a more refined classification of platelet dysfunction, including cases in which genetic findings or aggregation-based assays fail to define a clear abnormality.

Ultimately, decoding platelet identity via flow cytometry supports a shift from method-centered diagnostics toward phenotype-driven interpretation of platelet disorders. By enabling detailed, integrated assessment of all major stages of platelet function in a single analytical framework, flow cytometry enhances disease stratification, guides targeted genetic investigations and advances our understanding of the functional diversity underlying thrombocytopathies.

Inflammatory Chemokine Receptors CCR1 and CCR2 on the Peripheral Blood Lymphocytes in Patients with Chronic Lymphocytic Leukemia

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Introduction. Chronic lymphocytic leukemia (CLL), the most common lymphoma/leukemia in adults, remains incurable. Two main forms of this B-cell neoplasm are distinguished: an indolent slowly progressing and an aggressive rapidly progressing disease, when patients survive no more than 3 years after the diagnosis. Several studies have associated the high Epstein Barr virus (EBV) DNA load in peripheral blood (PB) cells with poor overall survival of CLL patients and with the more aggressive course of the disease. We demonstrated earlier that the EBV infection of B cells, isolated from PB of healthy donors, up-regulates the inflammatory chemokine receptors CCR1 and CCR2. The aim of this work was to analyze the cell-surface expression of CCR1, CCR2, and the CLL negative prognostic marker CD38 in PB lymphocytes of CLL patients and to assess associations with the EBV DNA load and presence of the EBV transcripts in PB mononuclear cells (PBMCs).

Materials and Methods. 54 CLL patients prior the treatment have been analyzed for the expression of CCR1, CCR2, and CD38 on PB lymphocytes by flow cytometry, using the BD FACSAria IIIu and Diva8.2 software. The EBV DNA loads and presence of the EBV transcripts (LMP1, LMP2A, EBNA2, and BZLF1) in PBMCs were determined using a commercial quantitative PCR kit and reverse-transcription (RT) nested-PCR, respectively.

Results. The correlation analysis revealed correlations between the frequencies of the CD38-positive and the CCR1- and CCR2-expressing PB CD19+CD5+ lymphocytes ($r_s = 0.50$ and $r_s = 0.38$, respectively). EBV DNA (≥5 copies/10^5 PBMCs) has been detected in 38% of the patients. In the EBV-positive patients, the frequency of the CCR2-presenting CD19+CD5+ PB lymphocytes was increased significantly, while the proportion of the CCR2-expressing CD19-negative (T and NK) lymphocytes was significantly decreased, compared with the EBV-undetectable patients (<5 copies/10^5 PBMCs).

Conclusions. Chemokine receptors regulate migration of immune cells. Apparently, CCR2 promotes dissemination of CLL cells from circulation into ligand-rich lymphoid organs, and the lack of CCR2 on T and NK lymphocytes indicates impaired mobility of these immune cells. We suggest that the CCR2-signaling pathway may represent targets for development of anti-CLL-progression therapeutics.

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The role of interferon-alpha in thymocyte development

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Type 1 interferons (T1 IFN) are a group of cytokines consisting of several IFN-α subtypes and IFN-β, critical for regulating inflammation during immune responses. They are constitutively expressed in the thymic microenvironment. T1 IFN signaling through IFN-α receptor 1 (IFNAR1) is critical for thymocyte differentiation, thymic epithelial cell homeostasis and activation of thymic antigen-presenting cells. T1 IFN have also been indicated in thymic involution taking place during aging as well as during insults causing a temporary decrease in thymic size and function. Current studies have been focusing on the role of T1 IFN and have not been able to differentiate between the effects of IFN-α and IFN-β.

Application of flow cytometry to assess the presence of proinflammatory cytokines in the urine of pediatric patients with E. coli-induced urosepsis

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Introduction: Nearly 25% of sepsis cases originate from the urogenital tract. That is why urosepsis is defined as sepsis caused by infections of the urinary tract, including cystitis, or lower urinary tract and bladder infections, and pyelonephritis, or upper urinary tract and kidney infections. This clinical condition may develop as a result of an activation cascade that, among other effects, enhances cytokine synthesis and secretion. The presence of bacterial antigens in the urinary tract of a child stimulates a rapid immune response, the production of cytokines and nitric oxide, and the influx of neutrophils. From this perspective, this study aimed to assess the cytokine profile in patients with urosepsis using flow cytometry.

Material and methods: The analyses were performed using blood plasma and urine of 34 young patients (aged 4 months - 17 years) who developed urosepsis caused by E. coli ESBL (-). The assessment of IL-12p70, IL-1, IL-8, IL-2, IL-10, TNF-α, IFN-γ, IL-4, IL-6, and IL-17A concentrations was performed using a commercial assay kit (BD Cytometric Beads Array). The analysis was performed on BD AccuriTM C6, and the results were calculated using FCAP ArrayTM Software (ver. 3.0.1).

Results: Except for IL-12p70 and IL-2, the analyzed cytokines were present in both plasma and urine of the patients studied. Urinary IL-6, IL-8, IL-1β, and IL-17A concentrations (as opposed to IL-10) were significantly higher than plasma values. Moreover, there were no sex differences in the secretion of the studied cytokines at the studied time point.

Conclusion: The results indicate that anti-inflammatory processes may be triggered with a delay, leading to exacerbation of clinical symptoms. Interestingly, a high urine concentration of IL-8, which plays a chemotactic role towards neutrophils and may lead to increased oxidative stress, contributing to tissue damage and intensifying inflammation, was observed. The presence of IL17-A among urosepsis patients seems to be a novel finding, and it might be related to its antibacterial function.

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Session 7:
Flow Cytometry in Basic and Applied Science

Tumor Antigen Presentation Defects: Impact on Immunotherapy Response and Strategies for Therapeutic Modulation in Preclinical Models

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Introduction: Anticancer immunotherapy boosts patients' own immune system to recognize and eliminate tumor cells, and can provide long-term remission. Howiever, low response rates to immunotherapy indicate prevalent resistance, highlighting the need to define its determinants and to develop rational combination treatment strategies. We investigated the impact of tumor Ag presentation mechanism (APM) status on responses to dendritic cell vaccination (DCV) and anti–PD-1 therapy, and tested whether cyclophosphamide (CY) can augment APM function and thereby improve chemoimmunotherapy efficacy in syngeneic C57BL/6 LLC1 and GL261 models.

Material and methods: C57BL/6 mice syngeneic LLC1 (Lewis lung carcinoma) and GL261 (glioma) tumors were assessed for APM status in vitro and tumor microenvironment in vivo (FC, qPCR). Autologous DCV were prepared from bone marrow (Lutz et al.) and loaded with tumor lysate (15   g/mL) and LPS (1   g/mL). Tumor-bearing mice received 3 doses of DCV (s.c., 1 × 106/100 µL PBS) or anti-PD-1 (i.p., 200 µg/100 µL PBS); tumor growth and induced immune response were monitored (FC, qPCR). CY effect on tumor APM was assessed in vitro (0,1-100   M) and in vivo (90-140 mg/kg) (FC, qPCR). The efficacy of combined CY, DCV and/or anti-PD-1 treatment was evaluated by tumor growth, APM status, and immune responses (FC, qPCR).

Results: Impaired tumor APM (LLC1 model) was associated with the absence of CD8+ T-cell response and resistance to DCV and anti-PD-1 therapy, whereas intact tumor APM (GL261 model) was associated with an elicited CD8+ T-cell response and sensitivity to immunotherapy. CY activated APM in LLC1 and GL261 cells in vitro and in GL261 tumors in vivo, together with induction of CD8+ T-cell response. Combined CY, DCV and anti-PD-1 treatment improved LLC1 and GL261 tumor control, but complete regression and immune memory were achieved only in mice with APM-intact tumors (GL261 model).

Conclusion: Tumor APM functionality determines antitumor immune response and sensitivity to DCV and anti-PD-1 therapy. Assessment of tumor APM status may have predictive value for patients' response to immunotherapy. CY enhances tumor APM function and synergizes with DCV and anti-PD-1. However, APM-impaired tumors may require additional interventions to achieve optimal CY-driven APM activation and the benefits of combination therapy.

Detection and phenotypic characterisation of acetylcholine receptor (AChR)-reactive CD4⁺ T cells in myasthenia gravis using antigen-reactive T cell enrichment (ARTE) and multiparameter flow cytometry

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Generalized myasthenia gravis (MG) is a chronic, relapse-prone autoimmune disease driven by pathogenic autoantibodies, most often directed against the acetylcholine receptor (AChR). Although established treatments such as thymectomy can lead to clinical improvement, a substantial proportion of patients experience incomplete remission or disease relapse, and no validated immunological biomarkers exist to predict long-term outcomes after surgery. In parallel, new targeted therapies, including efgartigimod alfa (Vyvgart®), a neonatal Fc receptor antagonist, have recently entered clinical practice; however, biomarkers that reflect underlying immune mechanisms or predict long-term therapeutic response are likewise lacking. Recent studies in other chronic antibody-mediated autoimmune diseases have identified a population of autoreactive CD4⁺ T cells with an exhaustion-like phenotype (ThEx) that persists despite treatment while retaining B cell helper capacity, potentially explaining chronicity and relapse in antibody-mediated autoimmunity. Whether such ThEx-like autoreactive CD4⁺ T cells are present in MG, and how they are affected by thymectomy or emerging therapies, remains unknown. To address this, we applied antigen-reactive T cell enrichment (ARTE) combined with multiparametric flow cytometry to directly detect and phenotype rare AChR-specific CD4⁺ T cells ex vivo. ARTE was used to track the frequency and phenotype of AChR-reactive CD4⁺ T cells before and after efgartigimod therapy and thymectomy. In parallel, comprehensive peripheral T and B cell immunophenotyping was performed, and in thymectomy cases, T and B cell subsets were additionally analysed in thymic tissue.
Initial data show that AChR-reactive CD4⁺ T cells were present at higher frequencies in MG patients than in healthy controls and exhibited increased FOXP3 expression together with the inhibitory receptors PD-1 and TIGIT, while retaining a conventional T helper lineage, consistent with an exhaustion-like (ThEx) phenotype. Therapy-associated changes in the frequency and phenotype of AChR-reactive CD4⁺ T cells were observed, accompanied by alterations in peripheral T and B cell compartments following both efgartigimod therapy and thymectomy. Further in-depth phenotypic characterisation of peripheral and thymic immune cell subsets is ongoing. Overall, these preliminary findings demonstrate the feasibility of integrating autoreactive CD4⁺ T cell analysis with broader T and B cell immunophenotyping to explore immune signatures relevant to treatment response and disease course in MG.

Multicolour spectral flow cytometry of tumour immune microenvironment in a mouse breast cancer model

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Introduction: Tumour immune microenvironment (TIME) plays an important role in cancer progression and therapeutic response. Detailed characterisation of tumour-infiltrating immune cells is therefore essential for understanding mechanisms of antitumour immunity. Alphavirus-mediated IFNγ gene delivery previously demonstrated a significant reduction in tumour size in a murine breast cancer model. In this study, alphavirus therapy was combined with photodynamic therapy (PDT), a treatment based on photosensitizer administration and further activation with light in the treated area. This study aimed to analyse immune cell composition and functional phenotypes within the TIME after combined immunotherapy using multicolour spectral flow cytometry and high-dimensional data analysis.

Methods: Semliki Forest virus (SFV) vector was employed for IFNγ gene delivery, while chlorin e6 was used as a photosensitizer for PDT. BALB/c mice bearing orthotopic 4T1 breast tumours were treated with alphavirus therapy, PDT, or their combination. After treatment, the tumours were excised, weighed, and processed into single-cell suspensions. Isolated immune cells were stained with a 17-marker panel and analysed using the Cytek Aurora 5L spectral flow cytometer. The flow cytometry data were analysed using FlowJo (v10.0.7). High-dimensional analysis of the macrophage population was performed in R (v4.4.1) using the flowCore package.

Results: Combined PDT and SFV/IFNγ therapy reduced tumour weight by 87.5%, compared to 40% after either monotherapy. Spectral flow cytometry and high-dimensional analysis of tumour immune cell infiltrate revealed complementary immune modulation by the combined treatment. PDT primarily enhanced innate immune activation, increasing natural killer (NK) cells and maturation of dendritic cells (DCs). Both monotherapies reduced immunosuppressive $CD206^{+}$ M2-like macrophages, while inducing $iNOS^{+}$ and MHC IIhigh M1-like macrophages. Combined therapy amplified these effects, further increasing DCs, NKs and $iNOS^{+}$ macrophages, while counteracting potentially negative effects of monotherapies. For example, SFV/IFNγ restored MHC II expression that was reduced by PDT and reduced PDT-enhanced regulatory and helper T cell populations.

Conclusions: Multicolour spectral flow cytometry enabled comprehensive profiling of TIME remodelling induced by combined alphavirus and photodynamic therapy. Synergy between these approaches results in tumour immune cell reprogramming towards an immunostimulatory phenotype, leading to efficient breast cancer treatment.

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Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Immune Cell Subsets in Glioblastoma Microenvironment

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Background: Glioblastoma is a common, malignant brain tumor that is heavily infiltrated by both lymphoid and myeloid cell populations. Among tumor immune infiltrate there are microglia, the brain-resident myeloid cells that contain highly autofluorescent lysosomes and hinder the detection of many fluorescent signals in flow cytometric analysis. The dissociation of brain tissue, the heterogeneity of immune infiltrate and microglia autofluorescence pose major challenges in successful analysis of glioblastoma microenvironment by flow cytometry

Methods: To study the immune landscape of glioma we used GL261-tdTomato+ intracranial glioma model. We optimized a dissociation protocol for tumor bearing brain tissue, including enzymatic digestion and gradient centrifugation, to optimally isolate and detect various immune cells infiltrating experimental mouse gliomas. We designed a 39-color spectral panel for the in-depth detection of various subsets of lymphocytes, macrophages, dendritic cells and NK cells using the Panel Builder in the Cytek Cloud. The panel included surface and intracellular antigens, as well as functional markers. Acquisition was performed using 5-laser Cytek Aurora. We took advantage of the autofluorescence extraction to identify major autofluorescence sources and use them for unmixing.

Results: We successfully identified over 20 immune cell populations in the experimental glioma microenvironment, including cDC1 and 2, CD49d+ bone-marrow derived macrophages, microglia, neutrophils, monocytes, intermediate monocyte-macrophage cells, CD3+ T cells, B cells, CD11b+ and CD11b- NK cells, NKT cells, CD25+ regulatory T cells, helper T cells and more. We obtained a high-quality detection of functional markers of myeloid cells such as Arg1, PD-L1, CD80, CD206 and MHC II, and lymphoid cells such as CD39, CD44/CD62L, TCF1, CD69, PD-1 and GITR. Data generated using the optimized protocol and spectral panel enabled unsupervised analysis using dimensionality-reduction methods and cell clustering.

Conclusions: Isolation of immune cells from tumor bearing brain requires an optimized protocol to achieve the efficient detection of their markers. Glioblastoma microenvironment requires autofluorescence extraction to yield a high-quality unmixing of spectral data. We present a multiparameter spectral panel that allows for an in-depth phenotyping of immune landscape of experimental mouse gliomas and can be broadly used in research and preclinical studies.

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Session 8:
Plenary session III

Powered by image-enabled spectral analysis: spatial protein localization and detection at the single cell level

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Flow cytometry has long served as a foundational technology in cell biology, providing high-throughput, quantitative data on cell size, granularity, and fluorescence. Despite its robustness, traditional flow cytometry is inherently limited by its inability to provide spatial context, often overlooking critical morphological details and complex structural features that define cellular function. Recent technological breakthroughs have bridged this gap by integrating high-resolution imaging with conventional flow systems. This hybrid approach, known as imaging flow cytometry, transforms cell analysis by capturing detailed qualitative morphological information alongside traditional quantitative metrics, offering a more holistic view of cellular populations.

A primary value of this integration lies in the precise subcellular localization of proteins within individual cells. Unlike standard conventional and spectral flow cytometry, which measures total fluorescence intensity, imaging capabilities allow researchers to locate exactly where a protein resides—whether it is expressed in the nucleus, bound to the plasma membrane, or localized within specific organelles. This spatial data is vital for understanding signaling pathways, protein translocation events, cell:cell interactions and cellular responses to external stimuli. Furthermore, the evolution of sophisticated sorting and analytical mechanisms measured at the speed of 10,000 events per second, based on these spatial parameters has opened new frontiers in multi-omics.

How AI is driving the future of flow cytometry and regenerative medicine

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Introduction: Artificial intelligence (AI) is increasingly integrated into flow cytometry and regenerative medicine to address the growing complexity of single cell data and the demand for scalable, reproducible, and clinically translatable cell therapies. Traditional analytical approaches struggle with high-dimensional datasets and variability inherent in biological systems. AI offers powerful tools to enhance data interpretation, automate workflows, and support predictive decision making across research and clinical applications.
Methods: This abstract synthesizes recent advances in AI driven flow cytometry and their application to regenerative medicine, drawing on developments in machine learning, deep learning, and data integration frameworks. Key methodological areas include automated cell population identification, unbiased clustering, anomaly detection, and multimodal data fusion combining cytometry with genomic, transcriptomic, and proteomic data. Emerging applications in real time analysis, adaptive experimental design, and closed-loop manufacturing systems are also considered.
Results: AI-based analytical methods significantly outperform manual and rule-based cytometry analyses by improving accuracy, reproducibility, and sensitivity, particularly for rare or transitional cell populations. In regenerative medicine, AI enhanced flow cytometry enables robust characterization of stem and progenitor cells, prediction of differentiation trajectories, and assessment of cell identity, potency, and safety. Integration of AI with automated cytometry platforms supports real time decision making and quality control during cell sorting and manufacturing, accelerating process optimization and reducing human bias.
Conclusions: AI is a transformative force in the convergence of flow cytometry and regenerative medicine. By enabling high resolution, scalable, and predictive cell analysis, AI addresses critical bottlenecks in data interpretation and cell therapy manufacturing. These advances support the development of personalized, reliable, and regulatory compliant regenerative treatments. As AI driven “smart” cytometry platforms continue to evolve, they are expected to play a central role in next generation biomedical research and the clinical translation of advanced therapy medicinal products.

Expanding the use of flow cytometry – application in non-hematopoietic body fluids

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Introduction: Flow cytometry is increasingly recognized as a valuable diagnostic tool for the evaluation of non-hematopoietic body fluids, where conventional cytology often demonstrates low sensitivity. Its application to cerebrospinal fluid (CSF), bronchoalveolar lavage fluid (BALF), EBUS-TBNA aspirates, fine-needle aspirates (FNA), effusion fluids, and vitreous samples improves the detection of neoplastic, inflammatory, and immunological processes in various anatomical locations. The growing clinical demand for rapid and precise immunophenotyping has strengthened the role of flow cytometry in complex diagnostic pathways.
Methods: This review summarizes sample handling, preparation, and immunophenotyping methods specific to each sample. Key methodological aspects include preanalytical stabilization of delicate samples such as CSF, standardized BALF processing, and customized antibody panels for EBUS-TBNA and FNA samples. Flow cytometry is also used for low-cellularity fluids, including vitreous samples, where it can be more effective than cytology. Immunophenotypic results are interpreted in conjunction with clinical, morphological, and molecular data to ensure diagnostic accuracy.
Results: In all fluid types studied, flow cytometry demonstrated significant diagnostic utility. In CSF, it enabled sensitive detection of central nervous system involvement in hematological malignancies and the identification of clonal lymphoid populations. BALF analysis contributed to the evaluation of interstitial lung diseases, infections, and eosinophilic diseases. EBUS-TBNA and FNA samples enabled rapid differentiation of lymphoid from epithelial proliferations, aiding the diagnosis of lymphomas and metastatic carcinomas. In vitreous samples, flow cytometry facilitated the differentiation of intraocular lymphoma from inflammatory conditions. These findings highlight the ability of this method to detect small, abnormal cell populations and its value in guiding further diagnostic steps.
Conclusions: Flow cytometry significantly improves diagnostic capabilities in non-hematopoietic body fluids, providing rapid, sensitive, and clinically relevant immunophenotyping. Despite challenges such as sample fragility, limited standardization, and the need for expert interpretation, it remains a valuable complement to cytology, histopathology, and molecular diagnostics. Its widespread use in various body fluids underscores its importance in the early detection of malignancies, characterization of inflammatory processes, and improved diagnostic accuracy in complex clinical situations.

POSTER ABSTRACTS

Immunomodulatory and cytotoxic effects of newly synthesized 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide derivatives on macrophages

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Background: Derivatives of 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide have demonstrated antitumor activity in cancer cell models. Given the central role of macrophages in regulating tumor-associated inflammation and immune responses, the present study evaluated the effects of six newly synthesized compounds (1–6) on macrophage viability, cell death pathways, and immunomodulatory phenotype.
Although derivatives of 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide exhibit antitumor activity in cancer cell models, their effects on immune cells within the tumor microenvironment remain insufficiently characterized. Macrophage function is closely regulated by NF-κB–dependent inflammatory signaling and L-arginine metabolism, which together determine polarization state and effector activity. This study investigated whether six newly synthesized derivatives (1–6) modulate macrophage survival and polarization-associated molecular pathways
Methods: Murine P388 macrophages were exposed to compounds 1–6 (25–200 μM) for 24 h and 72 h. Cell survival was determined using the MTT assay. Viability and mode of cell death were analyzed by flow cytometry with viability dyes in combination with Annexin V and propidium iodide. Macrophage polarization status was assessed immunocytochemically by evaluating expression of CD80 and CD206. Intracellular inflammatory responses were characterized by assessing phosphorylated NF-κB p65 as well as expression of inducible nitric oxide synthase (iNOS) and arginase 1 (Arg1).
Results: The tested derivatives induced concentration-dependent effects on macrophage survival, primarily through the induction of apoptosis. Importantly, sub-cytotoxic concentrations of selected compounds were found to significantly modulate macrophage activation states, as evidenced by alterations in surface marker expression and changes in metabolic polarization markers. These effects were accompanied by the modulation of pro-inflammatory signaling mediators.
Conclusions: The tested derivatives induce time- and concentration-dependent effects in macrophages, promoting apoptosis at higher concentrations while concurrently modulating macrophage activation and polarization-associated signaling. These findings indicate that the compounds may influence not only tumor cells directly but also immune components of the tumor microenvironment, supporting their further investigation as potential anticancer agents.
Funding: This research was funded by the European Union (NextGenerationEU) as part of the National Recovery and Resilience Plan, supported by the Medical Research Agency. Project number KPOD.07.07-IW.07-0072/24 (PI: J. Kulbacka). Acronym: Onko-SPARK.

Selective Electroporation-Enhanced Cytotoxicity of a Novel 4,6-Dimethyl-2-Sulfanylpyridine-3-Carboxamide Derivative in Human Melanoma A375 Cells

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Introduction: The search for novel anticancer agents with high efficacy and limited systemic toxicity remains a critical challenge in oncology. Recently synthesized 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide derivatives have emerged as promising candidates due to their cytotoxic and immunomodulatory properties. However, the intracellular activity of many small-molecule compounds is limited by inefficient membrane penetration. Electroporation (EP), a technique based on transient membrane permeabilization, represents an effective strategy to enhance intracellular drug delivery. Electrochemotherapy (ECT), combining reversible electroporation with chemotherapy, is currently limited in clinical practice to a narrow group of cytostatics, highlighting the need to identify new compounds suitable for EP-assisted oncological therapies.
Material and methods: Biological evaluation was performed using the human melanoma A375 cell line. Six novel 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide derivatives were analyzed for their cytotoxic activity with and without electroporation. Reversible electroporation was conducted using ESOPE-based parameters (1200 V/cm, 100 µs, 8 pulses, 1 Hz). Cell viability was assessed by MTT assay at 24 and 72 hours post-treatment. Electroporation-induced membrane permeabilization and recovery were examined using holotomographic microscopy, allowing real-time visualization of morphological changes following pulse application.
Results: Among the tested compounds, one derivative exhibited a pronounced and selective increase in cytotoxicity when combined with electroporation. A significant reduction in melanoma cell viability was observed as early as 24 hours post-treatment compared to compound exposure alone. The remaining derivatives did not demonstrate electroporation-dependent enhancement of cytotoxic activity. Holotomographic imaging revealed transient membrane blebbing immediately after electroporation, with complete recovery observed within approximately 30 minutes, confirming reversible membrane permeabilization. Electroporation alone did not significantly affect cell viability.
Conclusion: The study demonstrates that electroporation can selectively enhance the anticancer activity of a specific 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide derivative in human melanoma cells. The identified compound represents a promising candidate for electrochemotherapy and supports the concept of compound-specific screening in electroporation-based cancer treatment strategies. These findings provide a foundation for further pharmacological and immunological validation aimed at developing safer and more effective anticancer therapies.

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Analytical validation of the BD FACSDuet fully automated sample preparation versus the manual BD OneFlow workflow for routine hematologic flow cytometry diagnostics using lymphoid, plasma cell, and acute leukemia screening tubes (LST, PCST, and ALOT)

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Background: Automation of flow cytometry sample preparation promises enhanced efficiency, standardization, and traceability in hematologic diagnostics, yet its analytical equivalence to manual workflows remains underexplored. The LST, PCST, and ALOT BD OneFlow panels collectively address ~80% of routine laboratory requests for lymphoproliferative disorders, gammopathies, and acute leukemias.

Objective: This study aims to validate BD FACSDuet fully automated sample preparation against the manual BD OneFlow workflow.

Methods: The manual BD OneFlow workflow using dry antibody tubes (LST: lymphoid screening; PCST: plasma cell/gammopathy screening; ALOT: acute leukemia orientation) served as the clinical reference standard. Each panel was independently adapted to BD FACSDuet fully automated sample preparation with protocol-specific optimizations. Both manual and automated preparations were acquired on a BD FACSLyric cytometer. At the time of analysis, 91 patient samples were included (LST n = 46, ALOT n = 33, PCST n = 12).

Results : BD FACSDuet automated preparation demonstrated high analytical and diagnostic concordance with the manual BD OneFlow workflow across LST, PCST, and ALOT panels. In LST tubes (n = 46), $CD19^+$ B cells, $CD3^+$ T cells, and $CD3^-/CD19^-$ lymphoid populations showed strong correlations between automated and manual protocols ($r = 0.988$, 0.978, and 0.890, respectively; $p < 0.0001$), with Bland–Altman mean biases of 0,98 %, −2.4 % , and +0.82 %. B-cell light chains also showed excellent agreement (Igλ r = 0.993, bias −0.59%; Igκ r = 0.983, bias −0.56%). In PCST tubes (n = 12), $CD38^+CD45^+$, $CD38^+CD138^+$, plasma cell Igλ, plasma cell Igκ, and $CD19^+$ B cells showed strong correlations (all $r > 0.95$, $p < 0.0001$), with Bland–Altman mean biases of −0.11%, +0.08%, +0.08%, −0.01%, and +1.25% respectively. For the ALOT panel (n = 33), $CD34^+$/CD45^dim blast frequencies showed a very strong correlation ($r = 0.981$, $p < 0.0001$) with a low Bland–Altman mean bias of +0.54%. Quality control samples showed stable and reproducible results.

Conclusion : This study demonstrates that BD FACSDuet fully automated sample preparation is analytically and diagnostically equivalent to the manual BD OneFlow workflow for routine hematologic flow cytometry using LST, PCST, and ALOT panels.

Spectral flow cytometry-based profiling of B cells in peripheral blood and Peyer's patches in IgA nephropathy

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Background: Understanding the origins of galactose-deficient IgA1 (GdIgA1)-producing B cells is central to deciphering the pathogenesis of IgA nephropathy (IgAN). While circulating GdIgA1^{+} B cells have recently been identified in IgAN, the mucosal niches and differentiation pathways that give rise to these cells remain poorly defined. High-dimensional flow cytometry offers a powerful platform for interrogating these compartments in parallel.

Objectives: To develop a unified spectral flow cytometry workflow and harmonized multicoulor panel enabling matched, high-resolution B cell profiling in Peyer's patches and peripheral blood from IgAN patients and healthy controls.

Methods: We optimized a tissue-compatible mononuclear cell isolation protocol for intestinal biopsies containing Peyer's patches and established a parallel processing pipeline for matched peripheral blood. A unified spectral flow cytometry panel was designed to resolve B cell maturation, activation, and IgA-associated phenotypes, including detection of GdIgA1^{+} subsets. Data were analyzed using standardized gating and high-dimensional visualization tools to ensure cross-compartment comparability.

Results: The workflow enabled robust recovery of Peyer's patch-derived mononuclear cells and reproducible spectral flow cytometric acquisition across tissue and blood samples. The unified panel consistently identified naïve, antigen-experienced, and IgA-expressing B cell subsets, including CD27^{-}CD21^{+}IgA^{+} populations and GdIgA1^{+} antibody-secreting cells. Harmonized spectral signatures supported direct comparison of mucosal and systemic B cell phenotypes relevant to IgAN.

Conclusions: We present a standardized, tissue-compatible workflow and unified spectral flow cytometry panel that enable matched profiling of B cells across Peyer's patches and peripheral compartments in IgAN. This platform provides a methodological foundation for future studies aimed at dissecting B cell activation pathways and defining mucosal contributions to pathogenic IgA responses.

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High-performance double-emulsion droplet sorting enabled by Sony MA900 Cell Sorter

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[1] Samplix ApS

Conventional flow cytometry focuses on detecting 50orter50 markers or intracellular molecules at the single-cell level but often fails to capture key functional features, such as secreted proteins or 50or–50or interactions. The Samplix Xdrop system addresses this limitation by enabling droplet-based flow cytometry, where individual cells or 50or pairs are analyzed within millions of double-emulsion (DE) droplets. These droplets retain both the cells and their secretions, allowing functional analysis at the droplet level using standard flow cytometers. In this study, double emulsion droplets (65 pL) were generated and 50orter using the newly released sorting option on the Sony MA900 Cell Sorter. When DE droplets containing fluorescent beads were tested, sort recovery rates exceeding 90% and purity above 95% were achieved, without any need for manual adjustment of drop delay. By combining high-throughput droplet generation, compatibility with conventional instruments, and automated high-recovery sorting, a robust and scalable platform can be established for functional single-cell analysis and high-throughput screening using flow-cytometry.

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.

Influence of Cannabidiol-Halloysite Nanotubes Systems on Human Lung Carcinoma - In Vitro Study on A549 Cells

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Introduction: Lung cancer remains the leading cause of cancer-related mortality, with over 2.2 million new cases diagnosed each year and a five-year survival rate of less than 20%. Although cannabidiol (CBD) exhibits antiproliferative activity in lung cancer models, its therapeutic use is limited by poor solubility and bioavailability. Biocompatible halloysite nanotubes (HNTs) enable the encapsulation and sustained release of hydrophobic compounds. The impact of HNT-mediated CBD delivery on tumour growth and oxidative stress remains unclear.

Methods: CBD purity (>98%) was confirmed by HPLC. Human A549 lung adenocarcinoma cells were cultured in 2D monolayers and 3D spheroids, then treated with CBD, HNTs or a CBD–HNT formulation (1.5–40 μg/mL). Cell viability was assessed using the PrestoBlue assay. Spheroid growth and morphology were analysed using automated microscopy and image analysis.

Results: CBD significantly reduced cell viability in a dose-dependent manner, reaching ~5% at concentrations ≥8 μg/mL. HNTs alone affected viability only at the highest concentration tested. Co-treatment with CBD and HNTs resulted in higher cell viability than CBD alone, indicating reduced acute cytotoxicity. In 3D cultures, CBD inhibited spheroid growth and caused structural disintegration at higher concentrations. HNTs preserved spheroid integrity and the CBD–HNT formulation limited expansion while preventing disintegration.

Conclusion: In conclusion, CBD significantly decreased the viability of A549 cells in both 2D and 3D cultures, whereas HNTs mitigated cytotoxicity. The CBD–HNT formulation stabilised the structure of the spheroids and reduced acute toxicity, suggesting that HNTs could be used as nanocarriers for the controlled delivery of cannabinoids. These preliminary in vitro findings warrant further physicochemical characterisation and in vivo evaluation.

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Application of flow cytometry to assess the presence of proinflammatory cytokines in the urine of pediatric patients with E. coli-induced urosepsis

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Introduction: Nearly 25% of sepsis cases originate from the urogenital tract. That is why urosepsis is defined as sepsis caused by infections of the urinary tract, including cystitis, or lower urinary tract and bladder infections, and pyelonephritis, or upper urinary tract and kidney infections. This clinical condition may develop as a result of an activation cascade that, among other effects, enhances cytokine synthesis and secretion. The presence of bacterial antigens in the urinary tract of a child stimulates a rapid immune response, the production of cytokines and nitric oxide, and the influx of neutrophils. From this perspective, this study aimed to assess the cytokine profile in patients with urosepsis using flow cytometry.
Material and methods: The analyses were performed using blood plasma and urine of 34 young patients (aged 4 months - 17 years) who developed urosepsis caused by E. coli ESBL (-). The assessment of IL-12p70, IL-1, IL-8, IL-2, IL-10, TNF-α, IFN-γ, IL-4, IL-6, and IL-17A concentrations was performed using a commercial assay kit (BD Cytometric Beads Array). The analysis was performed on BD AccuriTM C6, and the results were calculated using FCAP ArrayTM Software (ver. 3.0.1).
Results: Except for IL-12p70 and IL-2, the analyzed cytokines were present in both plasma and urine of the patients studied. Urinary IL-6, IL-8, IL-1β, and IL-17A concentrations (as opposed to IL-10) were significantly higher than plasma values. Moreover, there were no sex differences in the secretion of the studied cytokines at the studied time point.
Conclusion: The results indicate that anti-inflammatory processes may be triggered with a delay, leading to exacerbation of clinical symptoms. Interestingly, a high urine concentration of IL-8, which plays a chemotactic role towards neutrophils and may lead to increased oxidative stress, contributing to tissue damage and intensifying inflammation, was observed. The presence of IL17-A among urosepsis patients seems to be a novel finding, and it might be related to its antibacterial function.

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Annexin Basophil Binding Assay (ABBA) in molecular diagnostics of wasp (Vespula spp.) venom allergy – a case study.

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Introduction: Hymenoptera insect stings are responsible for 48% of severe anaphylactic reactions occurring in European adults, and 20% of those reported in children [1]. A double positivity sensitization to bee and wasp venom extracts during in vitro testing occurs in up to 50% of venom-allergic patients [2]. The basophil activation test using flow cytometry may be an additional molecular tool to assess genuine versus cross-reactive sensitization to honey bee and wasp venom [3].
Material and methods: ImmunoCAP FEIA in vitro system was used to determine specific IgE against allergenic extracts of bee venom, wasp venom and rVes v5 allergenic component. Basophil activation test with 2 venom extracts was investigated in whole blood using flow cytometry.
Results: Presence of specific IgE antibodies against wasp venom allergen extracts and one component and honey bee venom: i3 (2.2 kUA/L), rVes v5 (20.0 kUA/L), i1 (1.0 kUA/L), serum tryptase level of 4.17 µg/L were observed. BAT with CD63 resulted in: 51.55% with yellow jacket venom extract and 0.8% basophil activity with honey bee venom extract in concentration 11.5 ng/ml. BAT with Annexin 5 resulted in: 65.24% with yellow jacket venom extract and 0.9% basophil activity with honey bee venom extract in concentration 11.5 ng/ml.
Discussion and conclusions: Allergy to wasp venom (class 3) were diagnosed based on ambulatory in vitro testing and medical history. In our case study, BAT with Annexin-5 proved to be comparable in effectiveness to that with CD63. On this basis, it would be advisable to continue, standardize and validate the study on a large group of patients to replace CD63 with a cheaper equivalent, Annexin-5.
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Load-Responsive Polymer Scaffolds for Targeted Extracellular Vesicle Release in Cartilage Repair: Flow Cytometry Study

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Background: Extracellular vesicles (EVs) derived from mesenchymal stromal cells (MSCs) are emerging as promising mediators of cell-free regenerative therapies due to their capacity to modulate inflammation and promote tissue repair. Menstrual blood–derived stromal cells (MenSCs) provide a non-invasive MSC source with strong proliferative and paracrine potential. In osteoarthritis (OA), articular cartilage experiences chronic mechanical stress, suggesting that therapeutic systems capable of responding to biomechanical forces may enhance treatment efficacy. Biomimetic scaffolds that replicate cartilage architecture may serve as reservoirs for controlled EV delivery; however, whether mechanical loading can regulate EV release from such materials remains largely unexplored.
Aim: To investigate mechanically induced release of MenSC-derived EVs from PCL-modified biomimetic scaffolds under conditions simulating osteoarthritic joint loading, using CFDA-based small-particle flow cytometry, and to evaluate scaffold cytocompatibility.
Methods: MenSCs were isolated from healthy donors and expanded in vitro. EVs were collected from conditioned media using size-exclusion chromatography. Scaffolds designed to mimic cartilage structure were surface-modified to enhance EV adsorption and stability. EV-loaded scaffolds were subjected to controlled mechanical compression, while parallel samples were maintained under static conditions. Released particles were collected at defined time points. Intact membrane-enclosed EVs were quantified by flow cytometry following labeling with carboxyfluorescein diacetate (CFDA), which becomes fluorescent after hydrolysis by intravesicular esterases, enabling detection of biologically active vesicles. Fluorescence triggering and calibrated reference beads were used to improve sensitivity for submicron particles. Appropriate buffer and dye-only controls were included to exclude background signals.
Results: Mechanical compression promoted increased release of CFDA-positive vesicles compared with static conditions, indicating load-dependent mobilization of intact EVs from modified scaffolds. Temporal analysis suggested sustained vesicle release over multiple days following stimulation.
Conclusion: Biomimetic scaffolds enable mechanically triggered release of biologically active MenSC-derived EVs. CFDA-based small-particle flow cytometry provides a sensitive approach for quantifying intact vesicles released from biomaterials, while cytocompatibility data support the suitability of this platform for regenerative applications. These findings highlight a load-responsive strategy for controlled, cell-free therapies targeting cartilage repair in osteoarthritis.
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Targeting CAIX Enhances Macrophage-Mediated Phagocytosis in Hypoxic PDAC-like microenvironment

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is one of the most severe malignancies, with a 5-year survival rate of only 8% . One of the factors contributing to its poor prognosis is the PDAC tumor microenvironment (TME), characterized by a dense, extracellular matrix rich stroma that creates a rigid tumor with compressed blood vessels (Hessmann et al., 2020). The lack of oxygen leads to hypoxia, which activates the expression of carbonic anhydrase IX (CAIX). Through its enzymatic activity, CAIX contributes to the acidification of the TME (Strapcova et al., 2020). An acidic TME not only drives the emergence of more aggressive tumor cell clones but also fosters an immunosuppressive milieu. In PDAC, macrophages represent the dominant immune cell population and strongly influence the overall immune response (Xu et al., 2021).
Aim: This study investigates how pancreatic cancer hypoxic TME impacts macrophage survival and whether CAIX-targeting antibodies enhance the capacity of antibody-dependent phagocytosis (ADCP) in control and tumor-derived media.
Methods: Colo357 pancreatic cancer cells were cultured under 1% O_2 hypoxia to generate 48-hour hypoxic conditioned medium (CM). THP-1 macrophage-like cells were then co-cultured with Colo357 cells. Phagocytosis was assessed by flow cytometry and confocal microscopy, with both cell lines labeled using membrane fluorescent dyes (CellBrite). In hypoxic conditions, Colo357 cells expressed CAIX, which was targeted by the humanized monoclonal antibody CA9hu-2 (Mabro) to evaluate ADCP.
Results: The CA9hu-2 antibody did not enhance phagocytosis under standard co-culture conditions, and THP-1 survival remained unchanged compared with the antibody-free control. Pre-exposure of THP-1 cells to tumor conditioned medium (TCM) reduced cell survival by 2-fold and phagocytic activity by ~30% during co-culture with Colo357 cells. Strikingly, in TCM-treated THP-1 cells, CA9hu-2 boosted phagocytosis by 45% and restored THP-1 survival to control levels.
Conclusions: Our experiments demonstrated that conditions simulating the hypoxic tumor microenvironment of pancreatic cancer markedly reduce both macrophage survival and their phagocytic capacity. Targeting the hypoxia-associated marker CAIX with a humanized CA9hu-2 antibody proved to be a beneficial approach, as it restored phagocytosis through activation of antibody-dependent mechanisms and simultaneously improved THP-1 cell survival.

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Meldonium-Associated Changes in Myocardial Macrophage Subsets and Inflammatory Markers in Experimental HFpEF model

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Introduction: Heart failure (HF) is associated with myocardial inflammation and increased cardiac macrophage presence, with roles in both injury and repair. Meldonium has shown cardioprotective effects in both experimental models and clinical settings. However, its impact on inflammatory signalling and immune cell remodelling in heart failure with preserved ejection fraction (HFpEF) remains poorly understood.
Methods: HFpEF was induced in male C57BL/6N mice through 16 weeks of a high-fat diet (HFD) combined with the hypertension-inducing agent L-NAME (0.5 g/L) in drinking water. Meldonium (200 mg/kg/day) was administered together with drinking water from week 9. At the end of treatment, cardiac function was assessed using echocardiography and invasive measurement of left ventricular pressure. After sacrifice, myocardial tissues were collected for qPCR analysis of inflammatory gene expression (TNFα, Il-1β, Il-6) and for quantification of tissue-resident macrophages, which were analysed by flow cytometry using antibodies against CD45-PerCP-Cy5.5, CD64-APC, MHCII-APC/Cy7, CCR2-BV421, and Ly6G-FITC.
Results: Echocardiographic analysis revealed normal systolic function across all groups. Mice receiving HFD and L-NAME developed left ventricular hypertrophy, which was further augmented by meldonium treatment. The HFpEF phenotype was characterized by elevated left ventricular end-diastolic pressure (8.1±1.4 mmHg), which was normalized by meldonium treatment (3.1±1.1 mmHg). qPCR analysis indicated mild inflammatory activation in the meldonium-treated group, with TNFα expression increased approximately 2-fold, Il-1β nearly 2-fold, and Il-6 by 4-fold. The proportion of immunoregulatory macrophages (MHCII^high CCR2^−) was higher in meldonium-treated mice (12%) compared with controls (4%) and untreated HFpEF mice (5%). Pro-inflammatory macrophages (MHCII^low CCR2^+) increased to 3.9% in the meldonium group versus 1.6% in controls.
Conclusions: Meldonium improved cardiac hemodynamic parameters in experimental HFpEF while simultaneously promoting myocardial inflammatory gene expression and macrophage subset remodelling. These results suggest that meldonium effects may result from coordinated metabolic and immune modulation.

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Flow Cytometric and Microscopic Evaluation of 5-Hydroxymethyluracil Expression and Leukocyte Marker Stability during Thermal DNA Denaturation

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Background: Thermal DNA denaturation is used in cytometric protocols for intracellular detection of selected DNA modifications (Gackowska et al., Methods Mol Biol, 2021); however, its impact on leukocyte surface antigen integrity remains poorly characterized. 5-Hydroxymethyluracil (5hmU) is an oxidative DNA base modification that requires effective DNA denaturation for reliable immunodetection. This study had two aims: (1) to microscopically and cytometrically evaluate intracellular 5hmU expression following thermal denaturation, and (2) to assess the stability of selected leukocyte surface markers under these conditions.
Methods: Whole blood samples were collected into TransFix® tubes from patients with chronic lymphocytic leukemia. DNA denaturation was performed by thermal treatment at 99 °C. Intracellular 5hmU was detected by indirect immunofluorescence using a rabbit anti-5hmU primary antibody followed by an Alexa Fluor 488 (AF488)-conjugated goat anti-rabbit secondary antibody. Samples were analyzed by fluorescence microscopy and flow cytometry. Control conditions included unstained samples and secondary-antibody-only staining. DAPI counterstaining was used to confirm intracellular and intranuclear localization of the 5hmU signal. In parallel, the effect of thermal denaturation on surface marker detection was assessed using antibodies against CD45, CD3, CD5, CD14, CD19, CD20, and CD38, representing different clones and fluorochrome conjugates.
Results: Fluorescence microscopy revealed strong, specific green nuclear fluorescence corresponding to AF488 exclusively in samples stained by indirect immunofluorescence. Co-localization of AF488 and DAPI signals demonstrated identical nuclear areas, confirming effective DNA denaturation and accurate intranuclear detection of 5hmU.
Flow cytometric analysis confirmed intracellular detection of 5hmU; however, thermal denaturation profoundly affected leukocyte surface marker stability. Among all tested antibodies, only CD45 conjugated with APC-Cy7 and BV421 retained measurable fluorescence signals, whereas all remaining surface markers failed to produce detectable signals after thermal treatment.
Conclusions: Thermal DNA denaturation enables reliable intranuclear detection of 5hmU but severely compromises the stability of most leukocyte surface markers. Anti-CD45 antibodies conjugated with BV421 (BD, Sony, clone HI30) and APC-Cy7 (Sysmex, clone 2D1) exhibit exceptional resistance to these conditions and may serve as reference markers in cytometric protocols combining thermal DNA denaturation with intracellular DNA modification analysis.
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Sex-Based Differences in Immunophenotype in Healthy Blood Donors

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Introduction: Immune reactions to a wide range of antigens—including harmless environmental antigens, self-antigens, tumor-associated antigens, as well as microbial and vaccine-derived antigens—exhibit marked differences between males and females. Growing interest in the biological basis of sex-related immune dimorphism has led to extensive research, with accumulating evidence indicating that sex hormones play a key role in shaping immune cell function. Therefore, we examined potential differences in basic immune phenotypes between males and females.
Material: Peripheral blood from 61 healthy blood donors (31 women and 30 men) was analyzed. Methods: Immune cell immunophenotyping was performed using standard IVD MULTITEST BD antibodies, and samples were analyzed by flow cytometry.
Results: The female group showed a higher percentage of lymphocytes (CD45++), T helper lymphocytes with a higher absolute count, as well as CD4/CD8 ratio, marginal zone-like B cells, class-switched B cells, and CD21low B cells than the male group. The male group was found to have elevated percentages of naïve B lymphocytes, transitional B cells, and plasmablasts. A weak positive correlation with age was found among double positive T lymphocytes, natural killer T cells (NKT) lymphocytes, and CD21low B cells. A negative correlation with age for double negative T lymphocytes, marginal zone-like B cells, and plasmablasts was noted.
Conclusions: The results indicated the importance of creating distinct reference ranges regarding sex and age concerning immunophenotype. Sex and age markedly influence immune cell composition in healthy individuals. Women exhibit higher T helper cell and memory B cell subsets, while men show elevated naïve and transitional B cells and plasmablasts. These differences highlight the need for sex- and age-specific reference ranges and suggest that therapies and interventions targeting the immune system—including immunotherapies, vaccines, and treatments for infections or autoimmune diseases—should consider these variations to optimize efficacy and safety.

Modeling kidney ischemia–reperfusion injury to investigate microRNA regulatory mechanisms

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Introduction: Ischemia-reperfusion injury (IRI) is a major cause of acute kidney injury (AKI) and contributes to progression toward chronic kidney disease. Renal IRI predominantly affects endothelial and proximal tubular epithelial cells. MicroRNAs (miRNAs) are important post-transcriptional regulators implicated in AKI pathogenesis and represent potential therapeutic targets. Human induced pluripotent stem cell (iPSC)-derived renal organoids constitute a promising in vitro model for studying renal injury mechanisms.
Methods: To provide a strong biological rationale for subsequent experimental work, a non-systematic literature review was conducted using PubMed, focusing on peer-reviewed studies addressing microRNA-regulated pathways in renal ischemia-reperfusion injury and acute kidney injury. Based on this background, the experimental part of the study was performed using human iPSC-derived renal organoids, which were allocated to control or hypoxia/reoxygenation (H/R) groups. IRI was modeled by 48 hours of hypoxia followed by 2 hours of reoxygenation. Cellular injury was assessed by spectrophotometric measurement of lactate dehydrogenase (LDH) activity released into the culture medium and normalized to total protein content. Renal cell identity and overall organoid architecture were evaluated by immunohistochemical (IHC) and immunofluorescence (IF) staining for the podocyte marker podocalyxin (PODXL) and the proximal tubule marker Lotus tetragonolobus lectin (LTL).
Results: The literature review identified multiple miRNAs involved in pathways related to apoptosis, inflammation, oxidative stress, and tubular injury in renal IRI. Experimentally, H/R-exposed renal organoids showed increased LDH release compared to controls, indicating enhanced cellular damage. IHC and IF analyses demonstrated that hypoxia/reoxygenation (H/R)-treated organoids retained characteristic podocyte (PODXL) and proximal tubular (LTL) markers, similar to those observed in control organoids.
Conclusions: Literature findings indicate a potential role of microRNAs in kidney ischemia–reperfusion injury. Preliminary experimental results support the applicability of iPSC-derived renal organoids for IRI modeling and future analyses of microRNA regulation.

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Revolutionizing Immunotherapy: Novel Strategies for In Vivo CAR-T Cell Generation and the Future of Cancer Treatment

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Introduction: Traditional chimeric antigen receptor (CAR) T-cell therapy has transformed the treatment of haematological malignancies. However, the current ex vivo manufacturing model has several limitations, such as high costs, complicated logistics and long production times ("vein-to-vein time"), which restrict patient access. This literature review focuses on recent advancements in generating CAR-T cells directly within the patient's body, which could represent a turning point in oncology.

Methods: A review of selected scientific publications regarding in vivo genetic engineering strategies was conducted. The efficacy and safety profiles of various delivery systems were analysed, including viral vectors (e.g. lentiviruses and adeno-associated viruses (AAVs)) and non-viral systems, with a specific focus on nanotechnology and lipid nanoparticles (LNPs) for mRNA delivery. Strategies for targeted delivery to T-lymphocytes were also evaluated.

Results: Literature analysis indicates that in vivo methods enable effective in situ T-cell transduction using targeted carriers (e.g. anti-CD3-conjugated LNPs). This approach eliminates the need for laboratory cell culture, drastically reducing treatment costs and timelines. The use of mRNA technology has been shown to enable transient CAR expression, mitigating the risk of long-term toxicity and adverse 'on-target, off-tumour' effects while maintaining potent antitumour activity in research models.

Conclusion: In vivo CAR-T cell generation is a promising alternative to conventional methods and has the potential to provide widely accessible, 'off-the-shelf' products. This technology could overcome the logistical and economic barriers of current therapies. Future research must focus on optimising gene delivery precision and confirming clinical safety, which could lead to improved access to advanced cellular therapies.

Platelets and platelet-derived extracellular vesicles can trigger cardioprotection?

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Introduction: Platelets are widely known for their detrimental effect on the myocardium during myocardial infarction (MI) and ischemia-reperfusion (IR) injury. This study investigated whether platelets, during chemical I/R, release platelet-derived extracellular vesicles (PEVs) and may protect cardiomyocytes from I/R injury.
Materials and methods: Platelets isolated from healthy human volunteers and cultured human cardiomyocytes (HCM) were subjected to a chemical I/R procedure consisting of 3 stages: stabilization, ischemia and reperfusion. The cells of the control group (HCM) and the study group (HCM contacted with platelets) were divided into 4 subgroups depending on the duration of ischemia. Analyses included platelet activation markers: GP IIb/IIIa, CD62P, CD63 and detection of PEVs (CD61 and CDIIb/IIIa) by flow cytometry, cardiomyocyte metabolic activity and inducible nitric oxide synthase (iNOS) tissue expression by fluorescence microscopy.
Results: Platelet activation markers increased during I/R. CD61 expression on PEVs surface increased with ischemia duration. Higher metabolic activity and reduced iNOS expression of cardiomyocytes compared to the control group were observed. Additionally, CD61 expression on the surface of PEVs correlated positively with cardiomyocytes metabolic activity.
Conclusions: Results suggest that under I/R conditions platelets and released PEVs may exert a cardioprotective effect. However further studies are needed to explain the mechanism of this phenomenon.

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Proteomic and transcriptomic signatures of unexplained infertility in menstrual blood mesenchymal stem cell extracellular vesicles

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Introduction: Unexplained infertility (uIF) affects about 1/3 of all infertile couples worldwide. Several biomolecules have been previously associated with uIF, mostly in peripheral blood and uterine biopsy samples, however the pathogenesis of uIF remains poorly understood. Menstrual blood is a non-invasive source of biomarkers representing uterine environment. The aim of this study was to analyse proteomic and transcriptomic signatures of uIF in menstrual blood mesenchymal stem cell-extracellular vesicle samples.
Methods: Menstrual blood was collected from 12 fertile healthy women and 8 women diagnosed with uIF. MenSC were isolated and characterized by mesenchymal marker expression using flow cytometry. MenSC-derived EVs were isolated from conditioned media using iodixanol density gradient ultracentrifugation. EVs were quantified using flow cytometry, and characterized according to the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines. CD9, CD63, CD81 and CD147 EV markers were detected by flow cytometry, intracellular EV marker TSG101 and contamination markers albumin, APOA1, APOB and APOE were analysed by Western blot. Transcriptomic and proteomic analysis of EV-associated proteins and microRNAs was conducted followed by bioinformatic analysis.
Results: MenSC from fertile controls and women with uIF showed similar mesenchymal phenotypes. EVs were successfully isolated and were characterized by MISEV criteria. Proteomic analysis revealed significant alterations in all three sample types from uIF patients, with enrichment of pathways related to impaired cell adhesion, immune and inflammatory responses, fibrosis, and altered metabolic processes. Transcriptomic analysis demonstrated a significant reduction of EV-associated miR-x and miR-y in uIF patients compared with fertile controls, suggesting impaired angiogenic signaling and dysregulated fibrotic remodeling within the endometrial microenvironment.
Conclusions: Integrated proteomic and transcriptomic profiling revealed differences between fertile and uIF groups, which could lead to early uIF diagnosis. Furthermore, proteomic signatures may serve as non-invasive biomarkers to define molecular endotypes of uIF.

Flow Cytometric Comparison of Different Human Myeloid Cell Enrichment Strategies

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Peripheral blood mononuclear cells (PBMCs) isolated via Ficoll density gradient centrifugation are widely utilized across immunological research, including cell-based therapeutic applications. PBMCs comprise T and B lymphocytes, NK cells, and monocytes in ratios that vary significantly between donors. The choice of enrichment method for specific cell populations can profoundly influence final yield, purity, and the potential for unintended cellular activation.
This study compares six myeloid cell isolation methods (plastic adherence, cold aggregation, density gradient centrifugation, immunomagnetic positive selection, immunomagnetic negative selection, suspension-based cell clumping, and a total PBMC control) to determine the optimal balance between myeloid enrichment, final cellular yield, and phenotypic integrity as evaluated by flow cytometry.
Our preliminary results suggest that physical selection methods provide cost-efficient and higher yields but at the expense of purity while immunomagnetic selection assays result in higher purity but lower absolute yields and an increased potential for myeloid cell activation.

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Clinical relevance of flow cytometric monitoring of B-cell depletion across disease conditions

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Introduction: Flow cytometry is a widely used technique for the identification and quantification of B-cell subsets in peripheral blood. In recent years, B-cell-depleting therapies targeting CD20 (e.g. rituximab, obinutuzumab, ocrelizumab) have become central to the treatment of autoimmune diseases, B-cell malignancies and post-transplant complications. Despite their broad clinical use, the role of systematic flow cytometric monitoring of B-cell depletion and repopulation remains heterogeneous across indications and clinical centers. This review summarizes current evidence on the diagnostic, prognostic and therapeutic relevance of flow cytometry-based B-cell monitoring in different clinical settings.

Methods: A narrative review of the literature focusing on studies published over the last 10-15 years was performed, with particular emphasis on B-cell depletion kinetics, subset analysis, prediction of treatment response or relapse, monitoring of immune reconstitution, and patient stratification in clinical practice.

Results: Evidence from multiple disease areas indicates that flow cytometry-based monitoring of B-cell depletion and repopulation provides clinically relevant information, including:

- association between B-cell repopulation patterns and relapse risk in selected autoimmune diseases such as rheumatoid arthritis, ANCA-associated vasculitis and multiple sclerosis;
- support for individualized retreatment strategies with anti-CD20 therapies based on B-cell kinetics in selected patient populations;
- assessment of immune reconstitution following hematopoietic stem cell transplantation;
- sensitive detection of minimal residual disease in B-cell malignancies;
- contribution to safety monitoring by characterizing immune status, in conjunction with immunoglobulin measurements, to identify patients at increased risk of immunodeficiency-related complications.

Conclusion: Flow cytometric monitoring of B-cell depletion and repopulation is a valuable yet variably implemented tool across clinical disciplines. When interpreted in disease-specific contexts and combined with clinical and laboratory parameters, it has the potential to improve patient stratification, inform treatment decisions and optimize long-term outcomes. Further standardization and prospective studies are needed to define its role across different indications.

Flow cytometry as a tool for testing bacterial physiology during the pyocyanin production process

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Pyocyanin is a phenazine produced exclusively by Pseudomonas aeruginosa and P. paraeruginosa. This redox-active compound has antibacterial and antifungal properties, which make it a potential biopesticide for agriculture. The production process requires optimized conditions that should be monitored. Flow cytometry can be used effectively to monitor the physiological state of cells, as it provides rapid results without the need for bacterial cultivation on agar plates.
This research aimed to use flow cytometry to monitor the viability and membrane stability of P. aeruginosa cells during pyocyanin production enhanced by zinc oxide nanoparticles.
Studies were conducted on P. aeruginosa ATCC27853 during the optimized pyocyanin production process enhanced by zinc oxide nanoparticles. Bacterial viability (LIVE/DEAD) ratio and membrane potential were measured on the BD Accuri C6 Plus flow cytometer using the respective kits (Thermo Scientific). Spectrophotometric assays (DCFH-DA and resazurin) were used for comparisons. FCS Express software was used for cytometric data analysis, whereas spectrophotometric data was analyzed in Origin.
Results confirmed differences in bacterial physiology during the nanomaterial-stimulated and control processes. The presence of nanomaterials did not affect the results, which were consistent across methods. Furthermore, both assays indicate the potential to detect changes in membrane stability in the studied cells.
Flow cytometry can be used to monitor bacterial physiology during pyocyanin production stimulated by zinc oxide nanoparticles.

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The use of imaging flow cytometry to evaluate the mechanism of netosis in blood and pulmonary neutrophils in horses

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Introduction: NETosis is a specialized neutrophil response mechanism that leads to the release of extracellular NETs (neutrophil extracellular traps), composed of chromatin fibers and granule proteins. This process is an important component of the innate immune response, but its excessive or chronic activation promotes organ damage observed, among others, in lung diseases in humans and horses. There are several types of netosis, the basic ones being suicidal (involving chromatin decondensation and membrane disruption) and vital (occurring without cell lysis, with the neutrophil retaining its effector functions). Among the many methods of analyzing netosis in cells, imaging flow cytometry stands out.

Objective: Since netosis plays an important role in the course of equine asthma, the aim of this study was to investigate its activity in peripheral blood neutrophils and bronchoalveolar lavage fluid (BALF) using morphometric methods and extracellular DNA assessment.

Methods: Blood and BALF were collected from six asthmatic horses as part of the diagnostic procedure. The obtained cells were incubated in the presence of 20 nM Phorbol 12-myristate 13-acetate (PMA) and 5 uM A23187 for 3.5 hours at 37°C with the addition of 5% CO2. Unstimulated samples served as controls. Neutrophils were labeled using anti-DH24A antibody. The cells were then dyed with wheat germ agglutinin (WGA), Hoechst, and anti-MPO antibody. NETosis analysis was done using an Amnis ImageStream MKII imaging flow cytometer. Also, extracellular DNA, as a representation of NET formation, was measured using cell impermeable DNA binding dye (SYTOX Green).

Results and discussion: Based on the method of Zhao et al. 2015, a method for morphometric analysis of horse cells was developed, showing both characteristics of cells in suicidal and vital NETosis.
Additionally, since the cytometric analysis only showed cells before their disintegration, complementing the analysis with an assessment of extracellular DNA in the supernatant allowed for the collection of complete data. The studies confirmed the significant contribution of cells capable of netosis in the course of asthma in horses.

Conclusion: The use of flow cytometry with imaging is a modern method that allows for the assessment of changes in cell morphology and their quantitative analysis.

Optimization of flow cytometry-based detection of 5-hydroxymethylouracil in patients with chronic lymphocytic leukemia

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Introduction: In patients with chronic lymphatic leukemia (CLL), DNA damage and increased level of 5-hydroxymethyluracil (5-hmU) are associated with the oxidative stress. The aim of the study was to optimize an intracellular flow cytometry staining protocol for 5-hmU detection in CLL,with particular emphasis on determining the optimal concentrations of the primary and secondary antibodies.
Method: Peripheral whole blood collected in EDTA/TransFix® was obtained from CLL patients, in whom leukocyte subpopulations were analyzed. Samples were stained with a monoclonal mouse anti-human CD45 BV421-conjugated antibody, a primary rabbit anti-5-hmU antibody, and a secondary goat anti-rabbit AF488-conjugated antibody. Validation was performed using a multicolor flow cytometry technique(Attune Cytpix, Thermo Fisher Scientific). Primary antibody titration included 11 volumes ranging from 0.5 to 20 μL, while the secondary antibody was tested in 8 dilutions ranging from 1:10 to 1:1500. The content of 5-hmU was presented as the ratio of the median fluorescence intensity (MFI) of the test tube (primary + secondary antibody) to the MFI of the control tube (secondary antibody only).
Results: Granulocyte and lymphocyte populations were assessed separately. In lymphocytes, the highest ratio was observed at primary antibody volumes of 4-5 μl(MFI=15,66 SD±14,39, MFI=14,95 SD±13,82, p =0,055, Friedman test), while a decrease in the ratio was observed at 10μl (MFI=11,72 SD±9,75).In contrast, no statistically significant differences were observed among the tested primary antibody volumes in granulocytes.
In both lymphocyte and granulocyte population, the ratio decreased with increasing secondary antibody dilution, with the highest ratios observed at dilutions of 1:10, 1:25, and 1:50(lymphocytes:MFI=81,38 SD±71,43, MFI=24,68 SD±12,25, MFI=15,97 SD±3,95, p = 0,0411, granulocytes: MFI=27,23 SD±14,27, MFI=11,08 SD±6,52, MFI=5,70 SD±1,05, p =0,00557 , Friedman test, respectively).
Conclusion: Optimal staining was achieved using a primary antibody volume of 5μL and a secondary antibody dilution of 1:50, yielding the highest signal-to-background ratio. Given that some patients may develop pancytopenia despite a high burden of clonal B lymphocytes, optimization should be guided by the signal-to-background ratio in the lymphocyte population. This approach facilitates standardization of the staining procedure.

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FLxFlow-HDSpect: Building a Collaborative Infrastructure for Advanced Spectral Flow Cytometry in Portugal

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In recent years, spectral flow cytometry (FC) has emerged as a breakthrough approach significantly increasing the number of measurable parameters simultaneously, while enabling improved sample analysis, particularly in complex biological samples with dim or rare populations and/or high autofluorescence. To meet the growing demand for high-dimensional (HD) spectral flow cytometry in Portugal, we established FLxFlow-HDSpect -The Lisbon Center for Advanced Applications in Spectral Flow Cytometry. This initiative, financed by the LISBOA2030-2024-15 program, builds upon the FLxFlow - Lisbon Flow Cytometry Network, a collaborative effort among four major research institutions in Lisbon area: the Champalimaud Foundation, the Fundação GIMM - Gulbenkian Institute for Molecular Medicine, NOVA Medical School, and the Research Institute for Medicines. These institutions work together to enhance expertise, optimize resources, and promote technological advancement in FC. FLxFlow-HDSpect is committed to expanding access to cutting-edge spectral cytometry instrumentation, expert support, and high-level training. The center provides specialized services, including experimental consulting, panel design optimization, spectral sample acquisition, high-resolution cell sorting, and HD data analysis using advanced computational tools. These services are designed to assist researchers in performing high-quality experiments and obtaining detailed, accurate data. Additionally, FLxFlow-HDSpect will offer training programs to ensure that researchers and technicians remain at the forefront of the latest FC methodologies. The FLxFlow-HDSpect infrastructure has been integrated into the existing FLxFlow network, significantly expanding the range of accessible tools and expertise. By providing access to advanced spectral flow cytometry technology, the infrastructure enhances both research and translational applications. Researchers now have the opportunity to analyze complex biological systems with greater precision, leading to improved research outcomes and enhanced clinical diagnostics. The infrastructure also allows for high-dimensional analysis and the generation of more comprehensive datasets, making it an invaluable resource for various scientific fields. The newly established FLxFlow-HDSpect aims to bring considerable advancements to the field of flow cytometry in Portugal. This initiative highlights the importance of regional collaboration for optimizing resources, sharing expertise, and accelerating discoveries. By sharing this experience, we aim to inspire other research centers to establish similar networks, fostering innovation and advancing spectral flow cytometry.

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Role of Autophagy in the Trafficking of B7 Family Proteins

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Introduction: Colorectal cancer is one of the most common malignancies worldwide and the third leading cause of cancer-related deaths. Chemoresistance remains an unresolved problem in the anti-cancer treatment. Cancer cells that are resistant to the initiation of cell death may activate molecular pathways that do not only confer chemoresistance but may also modulate the expression of immune checkpoint proteins. One of such pathways could be autophagy, which is usually perturbated in cancer cells, and it also is known to participate in protein trafficking to plasma membrane.
Methods: For this study, we used qPCR and western blotting to select B7 family proteins according to their altered expression in chemoresistant sublines of HCT116, SW620, and DLD1 cells. To silence autophagy, we chose several ATG proteins from different steps of autophagy process and evaluated their expression after knockdown by shRNA using western blotting. The importance of autophagy-specific ATG proteins for the surface presentation of B7 family immune checkpoint proteins was assessed by flow cytometry.
Results: We have revealed differential expression of several transcripts of B7 protein family in HCT116 and DLD1 sublines, that had developed acquired chemoresistance to 5-fluorouracil. Treatment with 5-fluorouracil or oxaliplatin also modulated the levels of B7 protein-coding transcripts in these cells. Moreover, silencing of various ATG proteins was shown to affect the cell surface exposure of B7 family immune checkpoint proteins PD-L1 and ICOSLG.
Conclusion: Autophagy contributes to the trafficking of B7 family immune checkpoint proteins.

Modeling the immune tumor microenvironment in syngeneic 4T1 triple- negative breast cancer mouse model

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Triple-negative breast cancer (TNBC) is the most aggressive subtype responsible for highest breast cancer related mortality. Lacking hormone receptors it consequently lacks a standardized treatment regimen. As a result, there is an urgent need for further research to improve therapeutic strategies. Mouse models are valuable tools for studying cancer, however, their accuracy can be limited since the immune system plays a crucial role in tumor development. To address this, we hypothesized that injecting immunocompetent mice with syngeneic cancer cell spheroids could lead to formation of tumors with immune-enriched microenvironment in comparison to tumors generated using syngeneic cell suspension. Despite its potential, research on tumor modeling using spheroids remains limited, with most studies conducted in xenograft models. In this study, we used the 4T1 TNBC cell line and syngeneic BALB/c mice to compare these injection methods. Spheroid formation was optimized, and in vivo data reveal differences in tumor growth patterns and immune cell infiltration. Spheroid-derived tumors showed enhanced angiogenesis, a more aggressive EMT phenotype, and distinct immune alterations, including increased lymphoid infiltration, reduced myeloid content, and a shift of macrophages toward immunosuppressive states.Our findings provide insights into how tumor injection methods influence the tumor microenvironment in an immunocompetent setting. By using a syngeneic model, this study could help improve the relevance of preclinical breast cancer research, particularly in understanding immune-tumor interactions.

The characteristics of anticancer activity of new 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide derivatives on BxPC-3 cells

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Pancreatic cancers are among the most aggressive tumors. Due to late diagnosis and frequent limitations in surgical resection, patients' prognosis is poor. Furthermore, the currently used chemotherapy is associated with a high risk of developing drug resistance. Electroporation enables increased drug uptake into cells despite low drug concentrations. Therefore, electrochemotherapy could be a promising alternative to conventional chemotherapies.
The study aimed to characterize the anticancer potential of new compounds within the 4,6-dimethyl-N-(2-hydrazinyl-2-oxoethyl)-2-thiopyridine-3-carboxamide class and to evaluate the effectiveness of electrochemotherapy in BxPC-3 cells.
Two of six derivatives were highly cytotoxic against different cancer cell lines, including human pancreatic cancer cells BxPC-3, as determined by MTT assays. The cell death pathway was confirmed using an apoptosis/necrosis assay, caspase activity, and a comet assay. Furthermore, significant changes in gene expression, crucial for apoptosis and the cell cycle, were observed in real-time RT-PCR. Using flow cytometry, we showed that both ESOPE conditions and nanosecond electroporation effectively permeabilized the cellular membranes of BxPC-3 cells. However, the effect of combined electroporation and compound treatment remained unclear.
In conclusion, newly synthesized 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide derivatives exhibit high anticancer activity and may be used for further prostate cancer treatment.

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Design and validation of a multicolor flow cytometry panel for macrophage phenotyping in Acute myeloid leukemia patient samples

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Introduction. Tumor-associated macrophages (TAMs) represent one of the major immune cell populations within the tumor microenvironment and can be broadly classified into classically activated (M1) and alternatively activated (M2) phenotypes. Accumulating evidence indicates that TAMs, particularly M2 macrophages, promote tumor growth, invasion, metastasis, and therapeutic resistance through immunosuppressive pathways, including PD-L1/PD-1 signaling and indoleamine 2,3-dioxygenase (IDO1) expression. Consequently, TAMs have become a central focus of cancer-related research. Despite growing interest in TAM biology, standardized multicolor flow cytometry (FC) panels enabling simultaneous macrophage phenotyping and immune checkpoint assessment in clinical samples remain limited. The aim of this study was to develop and validate a FC panel for macrophage phenotyping and assessment of IDO and PD-L1 expression for use in future experimental applications.
Methods. A literature review was conducted to identify markers relevant to the target cell populations. Markers selected for myeloid lineage and monocyte/macrophage identification included CD45, HLA-DR, CD14, CD16, CD80, CD206, and CD163. In addition, immune checkpoint – related markers IDO1, PD-L1 (CD274), and PD-1 (CD279) were incorporated into the panel. Gating strategies were developed to enable population discrimination and quantitative analysis. Fluorochromes, including a viability dye, were selected using FluoroFinder. The panel was validated using cryopreserved, heparinized bone marrow (BM) and peripheral blood mononuclear cell (PBMC) samples obtained from patients with acute myeloid leukemia, provided by the University of Bologna, Italy. The study was approved by the UL Ethics Committee (approval No. 71-35/59).
Results. The flow cytometry panel and gating strategy for the assessment of myeloid and macrophage immunophenotypes in BM and PBMC samples from patients with acute myeloid leukemia were successfully developed and validated.
Conclusions. This study presents a validated multicolor FC panel enabling macrophage immunophenotyping and evaluation of IDO1 and PD-L1 expression in AML patient bone marrow and PBMC samples, supporting its application in future TME studies.

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The impact of TLR7 pathway in murine thymic cell development

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The thymic microenvironment is characterized by constitutive, low-level sterile inflammation, including the steady production of Toll-like receptor (TLR)-induced cytokines. This tonic inflammatory signaling is thought to partially arise from endogenous ligands released from dying cells. While inflammation in the thymus is thought to be an integral component of thymic homeostasis, it is still poorly understood how innate inflammatory signaling pathways shape discrete thymocyte maturation stages and non-lymphoid thymic compartments.
In this study we investigate the impact of TLR7 signaling on distinct thymocyte subsets and thymic cell populations in C57BL/6 and AIRE-KO mice. Using both ex vivo fetal thymus organ culture and in vivo intrathymic delivery approaches, we modulate the inflammation in embryonic E16.5 and postnatal (3-4 week old) mouse thymi. After 4-5 days of stimulation with TLR7 ligands, functional changes are analyzed using 4-laser spectral flow cytometer with two 20-color panels, enabling us to analyse over 60 cell types from the lymphocyte, myeloid and stromal cell compartments.
Alterations were most evident within CD4 single-positive (SP) thymocyte compartments, where the augmentation of TLR7 pathway caused a shift from semi-mature towards more advanced maturation stages in both fetal and young thymi of C57BL/6 and AIRE-KO mice. In contrast, accelerated maturation of CD8SP thymocytes was detected selectively during C57BL/6 embryonic thymopoiesis. In addition, increasing inflammation through TLR-dependent signaling significantly reduced CD4-CD8- γδ T cells and increased DN thymocytes, a similar trend was observed in young thymi, suggesting bias toward conventional T cell differentiation. Beyond thymocytes, enhanced innate inflammatory signaling also reshaped the mTEC compartment in C57BL/6 mice, with selective expansion of mTEC I and III subsets and reduction in mTEC IV and mTEChi populations.
Overall our work identifies TLR signaling as an active regulator of thymocyte maturation timing, lineage bias, and stromal composition in the thymus. Manipulating the tonic inflammation in the thymus can open up new potential targets for modulating thymocyte development and thus the adaptive immune response.

The role of interferon-alpha in thymocyte development

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Type 1 interferons (T1 IFN) are a group of cytokines consisting of several IFN-α subtypes and IFN-β, critical for regulating inflammation during immune responses. They are constitutively expressed in the thymic microenvironment. T1 IFN signaling through IFN-α receptor 1 (IFNAR1) is critical for thymocyte differentiation, thymic epithelial cell homeostasis and activation of thymic antigen-presenting cells. T1 IFN have also been indicated in thymic involution taking place during aging as well as during insults causing a temporary decrease in thymic size and function. Current studies have been focusing on the role of T1 IFN and have not been able to differentiate between the effects of IFN-α and IFN-β.

Inhibition and Analysis of Cellular Senescence-Related Proteins in Osteoarthritic Cartilage and Synovium Extracellular Vesicles by Flow Cytometry

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Introduction: Osteoarthritis (OA) is the most common joint disease in the world, affecting millions of people globally. It is a complex degenerative disease encompassing many factors contributing to impaired cartilage regeneration and chronic inflammation, one of which being cellular senescence. A regulator of senescence – gap junction protein Connexin 43 (Cx43) may influence OA progression and in turn affect other cellular proteins and molecules. Targeting Cx43 with specific peptide inhibitors (TUB1) may potentially prolong cellular function, halt senescence and prevent its damage to the surrounding cells and tissues. This study analyzed the effects of TUB1 on extracellular vesicles (EVs) from cartilage and synovium samples, aiming to assess the effectiveness of Cx43 inhibition in the context of potentially treating or mitigating OA.
Methods: Following surgical procedures and joint replacement, cartilage and synovium tissues were obtained from hospitals, afterwards preparing them into tissue explants and culturing in vitro. The Explants were treated with Cx43 inhibitor TUB1, and later analyzed by cellular proliferation, glycosaminoglycan (GAG) deposition, cytokine secretion and gene expression. EVs isolated from explants by size-exclusion chromatography (SEC) were characterized for protein expression (Cx43, Pan1, CypA), evaluating the effects of TUB1 treatment by flow cytometer Cytoflex LX.
Results: TUB1 treatment resulted in reduced cellular senescence, validated by cellular β-galactosidase activity and flow cytometry. GAG release into cell medium also decreased, with an observed increase in extracellular matrix content (histological staining). Cytokine secretion after TUB1 treatment was affected, suggesting its effects on immunomodulation. Cx43 expression in extracellular vesicles also decreased, showing a notable result in cell EVs outside of the explants themselves. TUB1 may have a potential role in OA signaling and mediation, altering the cellular environment through Cx43 inhibition.
Conclusion: This study was conducted within the Horizon EU TWINFLAG project involving IMC, UCD, and UVigo, providing novel insights into the role of Cx43 in osteoarthritic cartilage and synovium samples and EVs. These results suggest that the peptide inhibitor TUB1 may be a potential candidate in future therapeutic applications, with the goal of mediating or inhibiting cellular senescence in OA progression.

Inflammatory Chemokine Receptors CCR1 and CCR2 on the Peripheral Blood Lymphocytes in Patients with Chronic Lymphocytic Leukemia

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Introduction. Chronic lymphocytic leukemia (CLL), the most common lymphoma/leukemia in adults, remains incurable. Two main forms of this B-cell neoplasm are distinguished: an indolent slowly progressing and an aggressive rapidly progressing disease, when patients survive no more than 3 years after the diagnosis. Several studies have associated the high Epstein Barr virus (EBV) DNA load in peripheral blood (PB) cells with poor overall survival of CLL patients and with the more aggressive course of the disease. We demonstrated earlier that the EBV infection of B cells, isolated from PB of healthy donors, up-regulates the inflammatory chemokine receptors CCR1 and CCR2. The aim of this work was to analyze the cell-surface expression of CCR1, CCR2, and the CLL negative prognostic marker CD38 in PB lymphocytes of CLL patients and to assess associations with the EBV DNA load and presence of the EBV transcripts in PB mononuclear cells (PBMCs).
Materials and Methods. 54 CLL patients prior the treatment have been analyzed for the expression of CCR1, CCR2, and CD38 on PB lymphocytes by flow cytometry, using the BD FACSAria IIIu and Diva8.2 software. The EBV DNA loads and presence of the EBV transcripts (LMP1, LMP2A, EBNA2, and BZLF1) in PBMCs were determined using a commercial quantitative PCR kit and reverse-transcription (RT) nested-PCR, respectively.
Results. The correlation analysis revealed correlations between the frequencies of the CD38-positive and the CCR1- and CCR2-expressing PB CD19+CD5+ lymphocytes ($r_s = 0.50$ and $r_s = 0.38$, respectively). EBV DNA (≥5 copies/10^5 PBMCs) has been detected in 38% of the patients. In the EBV-positive patients, the frequency of the CCR2-presenting CD19+CD5+ PB lymphocytes was increased significantly, while the proportion of the CCR2-expressing CD19-negative (T and NK) lymphocytes was significantly decreased, compared with the EBV-undetectable patients (<5 copies/10^5 PBMCs).
Conclusions. Chemokine receptors regulate migration of immune cells. Apparently, CCR2 promotes dissemination of CLL cells from circulation into ligand-rich lymphoid organs, and the lack of CCR2 on T and NK lymphocytes indicates impaired mobility of these immune cells. We suggest that the CCR2-signaling pathway may represent targets for development of anti-CLL-progression therapeutics.

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Assessment of 5-hydroxymethyluracil in peripheral blood leukocyte populations of chronic lymphocytic leukemia patients

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In chronic lymphocytic leukemia (CLL) patients, 5-hydroxymethyluracil (5-hmUr) in the DNA of PBMCs are 10–30 times higher than in healthy individuals and strongly correlates with Rai stage. Our goal was to assess the applicability of flow cytometry for evaluating 5-hmUr expression in lymphocytes, monocytes, and granulocytes of CLL patients.
Material and method: Peripheral blood collected in EDTA/TransFix® tubes from 19 untreated/observed and 18 treated CLL patients (Department of Hematology Jan Biziel University Hospital No. 2 in Bydgoszcz) was direct (CD45) or indirect (5-hmU) stained with antibodies using a previously developed protocol. The content of 5-hmUr was calculated as the ratio of the median fluorescence intensity (MFI) over the negative control.
Results: In both groups, the highest 5-hmUr ratio was observed in lymphocytes. In untreated/observed patients, a significantly higher 5-hmUr ratio was also found in the monocytes compared to granulocytes. The 5-hmUr ratio in the lymphocytes of treated patients was lower than in lymphocytes of the untreated/observed patients. A similar trend was observed in the monocytes. The 5-hmUr ratio in lymphocytes positively correlated with the percentage of B cells, helper T cells, and CD23 and CD200 density on B cells, and negatively with CD10 density. In monocytes, 5-hmUr ratio showed moderate positive correlation with percentage of lymphocytes as well as percentage of B cells expressing CD5+ and CD11c+ and CD200 density. A negative correlation was also found with granulocyte and NK cell percentages, as well as B cell CD10 density. A similar negative correlation with CD10 was observed in granulocytes 5-hmUr ratio.
Conclusion: As demonstrated in our study, flow cytometry enables rapid assessment of 5-hmUr expression at the cell population level. We showed that the highest 5-hmUr ratio occurs in lymphocytes, while the lowest are found in peripheral granulocytes. We also observed a significant reduction in 5-hmUr ratio in treated patients, as well as a positive correlation between 5-hmUr ratio in lymphocytes and the percentage of peripheral B cells. The results require detailed analysis, also in the context of patients' clinical data. With an expanded study group, they may provide valuable insights into CLL development and kinetics.

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Translational integration of routine flow cytometry methods: from diagnostics to ATMP manufacturing support

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Introduction: The Laboratory of Immunology at the National Cancer Institute (Vilnius, Lithuania) uses routine flow cytometry to connect clinical diagnostics with ATMP manufacturing and release for translational oncology. We collaborate with international commercial partners on EDIM test, with Loyola University Chicago on anti CD19 CAR T protocol adoption, and with local ATMP manufacturers on GMP aligned ATMP QC. We also optimize analytical readouts and manufacturing steps for implementation in our GMP classified cleanroom at NVI.

Methods: A harmonized workflow (validated multicolor panels, daily instrument performance checks, compensation controls, SOP based analysis, and traceable reporting) supports four workstreams. (1) Cryopreservation impact on clinical grade dendritic cell (DC) vaccines: viability recovery and phenotype are quantified before and after freeze–thaw; a comparability study evaluates CryoSURE-DEX40 (current manufacturing medium) versus other commercial cryomedia and a conventional 10% DMSO formulation under standardized freezing and thawing conditions. (2) EDIM: peripheral blood monocyte/phagocyte subsets are enumerated and assessed by intracellular staining of tumor associated epitopes to support clinical triage. (3) Routine ATMP QC: flow cytometric identity, purity, viability and subset composition testing is performed for DC products, cytokine induced killer cells, and other cell based therapies produced by partners, aligned with batch release criteria. Analytical performance is externally benchmarked through INSTAND (haematology/lymphocyte differentiation). (4) CAR-T protocol adoption: the Loyola anti CD19 workflow is transferred with in process and final product flow readouts (transduction efficiency, CD4/CD8 balance).

Results: In routine EDIM testing, our current implementation shows approximately 80% correct predictions when compared with downstream clinical diagnosis/work up. For ATMP QC, we have supported release decision making for over 10 years with Froceth -the only commercial ATMP manufacturer in the Baltics- and currently test more than 100 ATMP products per year, providing rapid, documented flow results that are integrated into partner release packages. Cryopreservation comparability data are being generated to inform a robustness oriented freezing strategy, and CAR-T analytical panels and reporting templates have been established for pilot runs.

Conclusion: Translational integration of routine flow cytometry across diagnostics, ATMP QC, and protocol adoption provides quality assured platform that strengthens regional cell therapy capacity and accelerates GMP readiness in Lithuania.

Alterations in Cytotoxic T Lymphocyte Subsets and their association with 5 hmU in Patients with Chronic Lymphocytic Leukemia

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Background: Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults in the Northern Hemisphere. Cytotoxic T lymphocytes (Tc) are crucial for antitumor immune surveillance, but the presence of a malignant leukemic clone can lead to their functional exhaustion and impaired immune response.
Objective: The aim of this study was to perform a quantitative and functional assessment of Tc lymphocytes subsets in CLL patients and analyze their associations with 5-hmU levels.
Materials and Methods:
TransFix/EDTA peripheral blood from twenty-five individuals were included: 13 untreated CLL patients under observation (median age 75 years) and 12 healthy controls (median age 84 years). Tc cell subpopulations were assessed by flow cytometry using anti-CD45 ECD, -CD3 KRO, -CD8 BV650, and -CD161 APC-AF750 antibodies. Additionally, intracellular expression of granzyme B (x-conjugated), perforin, and 5-hmU (AF 488-conjugated) were measured. Statistical analysis was performed using Mann–Whitney U and Spearman tests.
Results: The percentage of Tc lymphocytes expressing perforin was significantly increased in CLL (p=0.002), whereas p the mean/median fluorescence intensity (MFI) of perforin and granzyme B was reduced (p<0,0001). The pool of CD161high Tc cells was increased (p<0,0001), but their CD161 MFI was reduced (p=0.03), CD161low Tc cells showed a decreased frequency (p=0.008) and reduced CD161 MFI (p=0.001). CD161medium Tc cells also exhibited lower CD161 MFI (p=0.02). Additionally, Tc cell granzyme B MFI positively correlated with monocyte 5-hmU MFI, (r=0.407, p=0.048), whereas the percentage of granzyme B-expressing Tc cells negatively correlated with granulocyte 5-hmU MFI (r=−0.404, p=0.051).
Conclusions: These findings indicate dysregulation of Tc cytotoxic function in CLL, with both quantitative and qualitative changes in perforin, granzyme B, and CD161 expression. The simultaneous increase in perforin-positive Tc cells, despite reduced perforin MFI and reduced granzyme B expression, , suggests a partially preserved cytotoxic potential, possibly representing an early stage of functional impairment. Associations between 5-hmU levels and Tc cytotoxicity suggest oxidative stress that may modulate immune responses in CLL.

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The influence of new derivatives of 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide on the type of cell death in the MCF-7 breast cancer cells in oncological therapies

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Introduction: Breast cancer ranks among the top three most common malignant neoplasms in the world. Chemotherapy is a popular treatment for various types of cancer, including breast cancer. The project aims to conduct studies validating the anticancer activity of new compounds from the 4,6-dimethyl-N-(2-hydranizyl-2-oxoethyl)-2-thiopyridine-3-carboxamide group. In this study the effects of chemotherapy were evaluated using several novel anticancer drugs on MCF-7 breast cancer cells.
Methods: MCF-7 human breast adenocarcinoma cells were incubated with two new derivatives of 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide, C2 and C5 novel drug candidates at concentrations of 50 µM and 100 µM for 24h. The type of cell death after the applied therapy was determined by TUNEL, comet assay, and flow cytometry (FACS) methods.
Results: The tested derivatives C2 and C6, after 24 hours of incubation, caused a cytotoxic effect in the tested MCF-7 cells. The number of apoptotic cells increased with increasing drug concentrations. At a concentration of 100 µm, the number of apoptotic cells was over 90%. The number of apoptotic cells was similar after treatment with both C2 and C6 drugs.
Conclusions: The obtained results confirmed the effectiveness of the investigated therapeutic method. Heterocyclic derivatives exhibit significant anticancer activity against MCF-7 breast cancer cells, often inducing apoptosis via mitochondrial pathways. The project aims to develop new, safer anti-cancer drugs that minimize the side effects of current treatment methods and improve the improve the quality of life for cancer patients.
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Flow Cytometry at IN-MOL-CELL Platform: Capabilities and Applications

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High-throughput single-cell analysis is now essential in modern biomedical research. Consequently, flow cytometry and cell sorting are core technologies within the recently established IN-MOL-CELL research infrastructure at the International Institute of Molecular and Cell Biology (IIMCB) in Warsaw, Poland. The flow cytometry platform is closely integrated with advanced microscopy facilities, including confocal, super-resolution, and electron microscopy. This allows flow-based measurements to be complemented by imaging, providing deeper biological insight.

IN-MOL-CELL offers multicolor flow cytometry analysis and preparative workflows, including high-purity cell sorting into tubes and multi-well plates. Current instrumentation includes a CytoFLEX LX analyzer equipped with five lasers, a CytoFLEX SRT cell sorter with four lasers, and a BD FACSAria II with three lasers. Later this year, the platform will be expanded with the BD FACSDiscover S8, a five-laser spectral cell sorter with integrated imaging capabilities. Together, these systems support both routine and advanced applications, including high-parameter analyses, and enable improved identification of rare cell populations from challenging tissues.

Flow cytometry and cell sorting play a pivotal role in ongoing internal and external projects, including whole-genome CRISPR–Cas9 knockout screens with fluorescence-based readouts. The platform enables the isolation of rare cell populations from zebrafish tissues at multiple developmental stages to explore transcriptomic landscapes during tissue formation. It also supports label-free separation of liver stromal cells from healthy and treated mice for single-cell RNA sequencing. Additional applications include bead-based cytokines and chemokines quantification, assessment of cellular phagocytosis of fluorescent cargo in combination with microscopy, and routine immunophenotyping, cell cycle analysis, and viability assessment in cancer cell lines.

The IN-MOL-CELL flow cytometry unit operates in both full-service and user-access modes, provides extensive user training, and is open to academic and industrial collaborators.

Could CCR2+ Non-Classical Monocytes Serve as a High-Precision Diagnostic Marker for HER2-Negative Breast Cancer?

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Introduction: HER2-negative breast cancers, including triple-negative (TNBC) and aggressive luminal variants, necessitate the discovery of precise systemic biomarkers for early detection. Given that monocytes act as "systemic sensors" of oncogenic transformation, we investigated whether the immunophenotypic remodeling of monocyte subsets, specifically the atypical expression of CCR2, could serve as a diagnostic fingerprint for HER2-negative malignancies.
Methods: Peripheral blood monocyte architecture and CCR2 expression levels (percentage and MFI) were analyzed across classical (CD14++CD16-), intermediate (CD14++CD16+), and non-classical (CD14+CD16++) subsets in HER2-negative breast cancer patients and healthy controls. The diagnostic performance was validated using Receiver Operating Characteristic (ROC) curve analysis.
Results: Significant reorganization of the monocyte landscape was observed, characterized by a contraction of the total monocyte pool and a polarization toward the classical compartment, most notably in LB HER2(-) patients (87.13%). A robust, unidirectional upregulation of CCR2 was detected across all subsets. The most profound shift occurred within the non-classical compartment, where the frequency of CCR2+ cells expanded more than four-fold compared to controls [18.32% vs. 4.29%]. ROC analysis identified the percentage of CCR2+ non-classical monocytes as a superior diagnostic marker, yielding a sensitivity of 96% and a specificity of 96% (at a 9% cut-off). These shifts were independent of patient age, BMI, systemic inflammatory markers (hsCRP, CCL2), and tumor-specific clinicopathological features.
Conclusions: Early-stage HER2-negative breast cancer induces a distinct systemic "fingerprint" through the induction of CCR2 on non-classical monocytes. This phenotypic aberration, likely facilitating the recruitment of patrolling monocytes to the tumor microenvironment, offers extraordinary diagnostic potential as a highly sensitive and specific liquid biopsy marker.

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Mesoporous silica as a nanocarrier for cannabidiol

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Introduction: Cannabinoids, including cannabidiol (CBD), are the most thoroughly characterised group of secondary metabolites of Cannabis sativa L. CBD is credited with possessing a variety of pharmacological properties, including analgesic, anti-inflammatory and anti-cancer properties [1]. However, its poor water solubility and limited bioavailability restrict its use, necessitating the exploration of alternative delivery methods [2]. Using mesoporous silica as a nanocarrier for CBD is one possible solution.
Methods: The PubMed and Scopus databases were searched using the phrase ("CBD" OR "cannabidiol" OR "cannabinoids") AND ("mesoporous silica"). A total of 17 records were obtained. Only original research articles on the use of mesoporous silica as a CBD carrier were included. After excluding five duplicates and two review articles, five articles were included in the final analysis.
Results: All of the articles presented delivery systems for CBD based on mesoporous silica alone or in combination with other substances. Mesoporous silica enabled a higher drug loading than other carriers [3]. Using mesoporous silica improved the solubility and stability of CBD [3, 4]. Platinum-catalysed cannabidiol-loaded mesoporous silica nanoparticles encapsulated in chitosan exhibited significantly lower MMP-13 expression in rat osteoarthritis models [5], and CBD-loaded mesoporous silica nanocarriers demonstrated a higher mucosal penetration rate than plain CBD; adding propylene glycol further increased this effect [6]. Mesoporous silica-loaded amorphous CBD also exhibited higher relative bioavailability than crystalline CBD in in vivo piglet models [7].
Conclusions: Mesoporous silica is a promising nanocarrier for CBD as it improves solubility, stability and bioavailability compared to traditional CBD formulations. The reviewed studies suggest that these delivery systems could enhance therapeutic performance and address the limitations associated with poor water solubility. However, the number of available experimental studies remains limited, particularly in cancer-related in vitro and in vivo models. Therefore, further well-designed, standardised research is necessary to evaluate the safety, efficacy and potential clinical applications of CBD-loaded mesoporous silica systems.

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Assessment of BTLA expression in peripheral blood cells

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Introduction: B- and T-lymphocyte attenuator (BTLA) is an inhibitory immune checkpoint receptor that plays an important role in regulating immune responses. Engagement of BTLA with its ligand, herpesvirus entry mediator, triggers inhibitory signalling through the downregulation of T-cell receptor signalling. BTLA is expressed on multiple immune cell types, most notably T cells, but is also consistently detected on B cells, dendritic cells, and other leukocytes. Here, we aimed to investigate the frequency of BTLA+ cells in the peripheral blood of healthy volunteers.

Methods: Peripheral blood from healthy donors (in total 15ml) was collected into EDTA-containing tubes. Red blood cells were lysed using ACK lysis buffer, then the cells were centrifuged and washed with PBS. Next, we performed surface marker staining with FITC-CD19 and APC-BTLA antibodies. Data were acquired using the NovoCyte Advanteon flow cytometer. All procedures performed in the study were conducted in accordance with the ethical standards of the institution and with the 1964 Declaration of Helsinki and its later amendments. In silico analysis of BTLA expression in healthy tissues was performed from publicly available data obtained from Human Protein Atlas dataset (HPA, GTEx, and FANTOM5).

Results: Based on bioinformatics analysis, we found that BTLA is highly expressed in tonsils, followed by lymph node, appendix, spleen, and thymus. Flow cytometry analysis of peripheral blood samples revealed that ~37% (37 ± 3%) of blood cells from healthy donors were $BTLA^+$. Interestingly, ~90% of $CD19^+$ cells, which constituted 12 ± 3% of total blood cells, were $BTLA^+$, corresponding to a total frequency of $CD19^+BTLA^+$ cells of approximately 11 ± 3%.

Conclusions: Consistent with previous reports, we demonstrated that BTLA is highly expressed in organs associated with immune function and that BTLA is present on approximately 90% of CD19+ cells. In addition, flow cytometry analysis revealed a high frequency of total BTLA+ cells in peripheral blood of healthy donors (37 ± 3%).

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Practical Aspects and Results of Extracellular Vesicle Analysis by Flow Cytometry

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Introduction: Extracellular vesicles (EVs) are nanoscale membrane structures released by most cell types and are considered promising biomarkers for diagnostics and disease monitoring. However, EV research is technically challenging due to their heterogeneity in size and cellular origin, co-isolation with non-vesicular particles, and the detection limits of standard flow cytometers. There is also a strong need for standardized EV measurement protocols across instruments to enable quantitative comparison of results between laboratories. This study aimed to develop and optimize a practical flow cytometry protocol for EV detection, characterization, and quantification according to MISEV standards, applicable in a core facility environment.

Methods: EVs were isolated by differential ultracentrifugation from both human serum samples and conditioned media of the HCT116 cell line. Measurements were performed using calibrated Cytek Aurora and Beckman Coulter CytoFLEX LX flow cytometers. Size calibration was conducted using NIST-traceable beads and the FCMPass software. EVs were labeled with fluorescent antibodies targeting canonical EV markers (CD9, CD63, CD8) and the membrane dye. Staining conditions, including antibody concentration, dye dilution, and incubation time, were systematically optimized.

Results: The optimized staining protocol enabled clear and specific detection of EVs across instruments. Antibody labeling provided reproducible fluorescence signals with minimal background, while specific membrane dye effectively distinguished vesicular from non-vesicular events. Calibration ensured consistent gating of EVs populations and facilitated cross-instrument comparability. The protocol demonstrated robustness for both serum-derived and cell culture–derived EVs, with reliable discrimination of CD9+, CD63+, and CD81+ subpopulations.

Conclusion: The developed protocol allows reliable identification and analysis of extracellular vesicles by flow cytometry following MISEV guidelines. The combination of optimized antibody staining, validated membrane dye, and rigorous calibration supports reproducible EV measurements across instruments. This workflow provides a practical framework for flow cytometry core facilities and users aiming to implement standardized EV analysis.

From antioxidant activity to immune modulation: functional evaluation of polyphenols in an in vitro inflammation model using flow cytometry

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Introduction: Polyphenols are a widely studied and structurally diverse group of plant-derived compounds highly abundant in fruits, vegetables, and medicinal plants. This study aimed to evaluate selected polyphenols and their derivatives, identified in the highest concentrations in plants or found in locally grown plant species, using a stepwise in vitro approach to assess their functional anti-inflammatory potential.
Methods: Antioxidant activity of phenolic acids and flavonoids was first assessed using the DPPH radical scavenging assay, and EC50 values were determined. Non-toxic concentration ranges for cellular experiments were established in murine RAW 264.7 macrophages using the MTT assay (up to 100 µM). Based on cytotoxicity profiles, appropriate concentrations were selected for subsequent immunological analysis. Macrophage activation was induced by lipopolysaccharide (LPS, 10 ng/mL) stimulation for 24 hours in the presence or absence of selected polyphenols. Flow cytometry was used to assess macrophage phenotype based on surface expression of the pro-inflammatory markers CD80 and CD86.
Results: Antioxidant activity varied substantially across the tested polyphenols. Among the phenolic acids, rosmarinic acid showed the highest antioxidant potency (EC50=11.0±2.7 µM), followed by caffeic acid (EC50=13.4±6.6 µM). Several flavonoids exhibited strong antioxidant activity with EC50 values below 10 µM, whereas others showed minimal or no radical scavenging activity under the tested conditions. MTT assay confirmed that compounds with strong antioxidant activity frequently exhibited increased cytotoxicity toward RAW 264.7 macrophages at higher concentrations. When evaluated at non-toxic concentrations, most flavonoids did not significantly affect LPS-induced macrophage polarization. In contrast, rosmarinic and caffeic acids significantly reduced M1 polarization at 100 µM, decreasing the proportion of $CD80^{+}CD86^{+}$ macrophages from 28.0±1.8% to 20.3±2.2% and 19.9±2.0%, respectively ($p<0.05$).
Conclusion: Flow cytometry-based phenotyping identified rosmarinic and caffeic acids as effective modulators of pro-inflammatory macrophage polarization, while all of the tested flavonoids were inactive. These results demonstrate that antioxidant capacity alone is not predictive of anti-inflammatory activity in a cellular immune model. This supports the use of functional immune assays and highlights the relevance of future studies exploring the combined effects of polyphenols in plant-derived systems.

Technological Advances in the Isolation and Detection of $CD45^{+}EpCAM^{+}$ Circulating Tumor Cells in Non–Small Cell Lung Cancer

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Introduction: Lung cancer is still the leading cause of cancer-related deaths worldwide. Non-small cell lung cancer (NSCLC) accounts for around 85% of cases. The late stage at which lung cancer is typically diagnosed highlights the urgent need for non-invasive biomarkers for early detection and treatment monitoring. Circulating tumour cells (CTCs), including the $CD45^{+}EpCAM^{+}$ subpopulation, play a key role in metastatic dissemination, epithelial-mesenchymal transition and immune evasion; therefore, they are promising candidates for biomarkers.

Methods: A narrative review of the literature was performed to evaluate technologies for isolation and detection of $CD45^{+}EpCAM^{+}$ CTCs in NSCLC. The analysed approaches included flow cytometry, which uses fluorochrome-labelled antibodies to analyse peripheral blood mononuclear cells (PBMCs) and tumour cells; immunomagnetic EpCAM-based enrichment, which uses the FDA-approved CellSearch platform; size-based filtration systems, such as ISET; hybrid platforms, which combine size filtration with immunofluorescence staining (DAPI/CD45/EpCAM); spiral microfluidic devices, which enable label-free enrichment based on flow dynamics; and selected molecular detection methods. Performance, sensitivity, recovery rates and clinical applicability were comparatively assessed.

Results: CTC abundance is consistently higher in NSCLC patients than in healthy individuals, supporting their diagnostic and prognostic potential. However, their extremely low frequency in peripheral blood remains a major technological limitation. CellSearch demonstrates limited sensitivity in NSCLC due to heterogeneous EpCAM expression. ISET enables the recovery of higher numbers of CTCs, but may miss smaller CTCs. Hybrid filtration–immunofluorescence platforms have demonstrated significantly higher CTC counts in stage IV patients than in stages I–III patients, indicating their prognostic relevance. Spiral microfluidic systems increased detection efficiency by 6–24 fold and enabled the concentration of enriched cells in small samples. Although flow cytometry enables multiparametric phenotyping, it requires effective pre-enrichment due to the rarity of CTCs. Molecular methods offer high analytical sensitivity, but limited morphological and phenotypic resolution.

Conclusions: CTC detection technologies differ substantially in analytical principles and performance. The lack of standardised protocols for blood collection, fixation, enrichment and analysis limits reproducibility and comparability between studies. The standardisation and validation of $CD45^{+}EpCAM^{+}$ CTC assays could facilitate their implementation in the clinic as reliable biomarkers for monitoring therapy and assessing disease dynamics assessment in NSCLC.

Substrate stiffness modulates radiation-induced DNA damage response in glioblastoma and microglial cells

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Mechanical properties of the cellular microenvironment are important regulators of cell processes like proliferation, spreading, migration, differentiation, and stress responses. Substrate stiffness affects chromatin organization, DNA repair pathways, and also cell fate, however its role in cell response for radiation induced DNA damage is still insufficiently characterized [1,2]. This is especially important in the context of brain irradiation, where therapeutic radiation affects both tumor cells and surrounding normal cells. In our research we investigated how substrate stiffness influences early DNA damage and cellular response to X-ray radiation in glioblastoma and microgial cells.

Human glioblastoma LN229 and microglial HMC3 cells were cultured on polyacrylamide hydrogels with different elasticity (0.2 kPa, 5 kPa, and 23 kPa) and on the glass surface. Cells were irradiated with 2 Gy or 8 Gy X-rays. Early DNA double-strand breaks were measured by immunofluorescent staining of γH2AX and foci analysis in ImageJ software. DNA damage after 8 Gy dose was assessed by comet assay whereas cellular response reflected in the cell cycle distribution and apoptosis was measured using flow cytometry.

Both cell lines showed increase in γH2AX foci following irradiation compared with non-irradiated controls. Comet assay confirmed elevated DNA damage after high-dose exposure. Cells grown on hydrogel substrates differed significantly from cells cultured on rigid glass in terms of DNA damage. However, no significant differences were observed among hydrogels in the tested stiffness range. Flow cytometric analysis of cell cycle distribution and apoptosis after 8 Gy irradiation is currently in progress.

Our results indicate that substrate mechanics modulate radiation-induced DNA damage responses, mainly at the level of rigid (glass) versus elastic growth conditions rather than within the selected hydrogel stiffness. Ongoing cytometric analyses will determine whether these mechanical effects extend to cell cycle arrest and apoptosis. The study highlights the importance of microenvironmental mechanics in radiobiology and suggest that substrate conditions may influence radiation responses of both glioblastoma cells and microglia.

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Flow cytometry in nanomaterial-contaminated bacterial cultures

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Flow cytometry is a powerful technique that surpasses classic methods for bacterial enumeration, such as the plate count method, developed in the late nineteenth century and still used in many laboratories. However, some environments contain contaminants that may hinder the proper analysis of flow cytometry results, giving the classic approach an advantage. On the other hand, flow cytometry, in addition to cell counting, can be used to assess the live/dead ratio. Therefore, this study aimed to test the utility of flow cytometry for nanomaterial-contaminated samples.
The biological material for the studies consisted of reference bacteria (Escherichia coli, Staphylococcus aureus, and Pseudomonas aeruginosa). Bacterial cultures were contaminated with selected nanomaterials (i.e., metal oxide nanoparticles or graphene oxide), and bacterial viability was further assessed by flow cytometry (BD Accuri C6 Plus with autosampler) using a mixture of Syto9 and Propidium iodide.
Results confirmed that flow cytometry could be used for viability analysis in contaminated samples. However, a significant restriction of the method has been determined. The main issue was the cell/nanomaterial ratio. Exceeding the nanomaterial threshold led to a loss of assay sensitivity. It could be improved by separating nanomaterial-cell agglomerates from planktonic cells and by rinsing the cells with physiological salt solution.
Flow cytometry can be used to study nanomaterial-bacteria interactions in nanomaterial-contaminated bacteriological media, although its use is restricted by the cell/nanomaterial ratio.

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Electroporation-modulated activity of novel 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide derivatives in A549 lung cancer cells

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The aim of this study was to investigate the anticancer activity of novel 4,6-dimethyl-N-(2-hydrazinyl-2-oxoethyl)-2-thiopyridine-3-carboxamide derivatives, including hydrazides and triazole- and oxadiazole-based compounds (C1–C6). These chemical scaffolds have attracted interest due to their pronounced biological activity and ability to interfere with pathways involved in cancer cell survival and proliferation. Previous studies indicate that triazole- and oxadiazole-based derivatives exhibit cytotoxic activity against lung cancer cells, including A549, and modulate oncogenic signaling such as EGFR [1,2]. A comprehensive biological, biophysical, and pharmacological evaluation of six investigated compounds (C1–C6) was performed using the human lung adenocarcinoma A549 cell line. Cell membrane permeabilization induced by micro- and nanosecond pulsed electric fields was assessed using YO-PRO-1 iodide (Sigma-Aldrich, Burlington, MA, USA) and analyzed by flow cytometry (CyFlow® Cube 6, Sysmex). Electroporation was applied in combination with different concentrations of the tested compounds to modulate cancer cell sensitivity to treatment. Cytotoxicity was evaluated by MTT and PrestoBlue assays at early and late time points. The results demonstrated enhanced cytotoxic effects of selected compounds when combined with electroporation. Electroporation increased membrane permeabilization and altered dose–response profiles. The magnitude and pattern of responses varied between compounds, suggesting that the interaction between compound structure, membrane permeabilization, and cellular susceptibility extends beyond increased drug uptake alone. In conclusion, 4,6-dimethylpyridine-3-carboxamide derivatives represent a biologically active class of compounds whose effects on cancer cells can be modulated by electroporation. These findings highlight the potential of combining chemical and biophysical approaches to tailor anticancer strategies and demonstrate the value of flow cytometry-based assessment in evaluating electroporation-mediated drug effects.

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Levelling the scale: Developing an integrative workflow for isolation of small particles by sorting

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Small particles (SPs) have gained increasing attention across multiple research fields for their crucial role in mediating intercellular communication. Still, the subcellular size of SPs hampers an accurate identification, due to the abundance of contaminants such as proteins or cell debris in the sample buffers. Flow Cytometry has emerged as a powerful tool for SPs characterisation, offering a multiparametric analysis at a single particle level. As such, in this work, we aimed to develop a sorting workflow for SPs enrichment, providing clearer fractions for characterisation and downstream applications.

As a case study for our approach, we employed a Cytek Aurora CS to sort extracellular vesicles and autophagosomes previously obtained from muscle cell lysates. Alongside the improved sensitivity of a violet side scatter detection, vesicles were stained with fluorochrome-coupled antibodies and detector gains were increased by 200% to enhance identification. A trigger threshold for the primary peak channel of each fluorochrome was also applied. Following MIFlowCyt-EV guidelines, controls for contaminant identification and single vesicle resolution were considered to better define populations of interest. To corroborate the purity of the sorted fraction, both non-sorted and sorted samples were analysed by Transmission Electron Microscopy (TEM).

Through serial dilutions of all samples, the coincident particle detection (swarm effect) could be mitigated, increasing the confidence of the acquired populations. The optimisation of the trigger threshold plus the detector gains avoided losing vesicles with lower fluorescence intensity, while enabling the separation from background noise. The populations of interest were further confirmed using buffer and reagent controls, minimising the sorting of cell debris and antibody aggregates. Data from TEM confirmed the enrichment in vesicles on the sorted fraction, which showed an average size range between 200 and 700 nm.

We have developed a suitable workflow for SPs enrichment, based on fluorescence signal detection. Sorting can be integrated with TEM, corroborating SPs enrichment, while providing additional information on particle size and morphology. This approach shows potential for different integrative workflows, providing more in-depth information for SPs and even assisting in the discovery of novel biomarkers or therapeutics.

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Spectral Flow Cytometric Evaluation of Tonsil Organoids Cultured in a GelMA-Based Extracellular Matrix

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Human tonsil organoids are a useful ex vivo model for studying adaptive immune responses, as they preserve lymphoid cell composition and enable analysis of B-cell activation, differentiation and antibody production. Insert-based tonsil organoid cultures, as described in Wagar et al. (2021), support GC–like organization and function. However, 3D extracellular matrix (ECM)–based culture approaches may complement this system by providing mechanical support and spatial confinement that more closely mimic lymphoid tissue microenvironment and may influence cell interactions and survival.
Here, we evaluated a GelMA-based ECM (LunaGel™) for tonsil organoid culture in comparison with the established insert-based protocol, focusing on antibody responses and immune cell population analysis using spectral flow cytometry.
Tonsil lymphoid cells, with or without 1% tonsil stromal cells, were cultured for 10-11 days embedded in ECM or on Millipore cell culture inserts. Organoids were stimulated with SARS-CoV-2 spike protein, IL-21, R848, or in combination. Antibody production was measured using a luciferase immunoprecipitation system (LIPS) assay targeting the SARS-CoV-2-RBD. Immune phenotypes were assessed at baseline and endpoint using a 4-laser Sony ID7000 spectral cell analyzer with a 15-antibody panel targeting lymphoid populations. Cells were recovered from ECM either by enzymatic digestion as per the manufacturer's protocol or by mechanical extraction, used in insert-based cultures.
ECM cultures showed limited organoid formation and did not support detectable naïve B-cell-mediated antibody responses. LIPS assays revealed memory-driven antibody production primarily in IL-21 stimulated conditions. Spectral flow cytometry consistently demonstrated loss of key immune markers in ECM cultures, including CD4, CD8, CD38, CXCR5, CD25, and CD20. This marker loss was reproducible and associated with enzymatic cell recovery. Notably, mechanical extraction from ECM matrices preserved immune marker signals, resembling insert-based cultures.
In conclusion, spectral flow analysis revealed that GelMA-based ECM culture and recovery methods can significantly compromise immune marker detection in tonsil organoid systems. While ECM culture supports cell survival, enzymatic recovery adversely affects epitope preservation, limiting immune profiling. Optimization of cell recovery workflows is essential before ECM-based tonsil organoids can be integrated into spectral flow cytometry driven immune analyses.

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Electrochemotherapy with Novel Drug Candidates Enhances Cytotoxicity in Human MCF-7 Breast Adenocarcinoma Cells

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Introduction: Breast cancer remains a leading cause of cancer-related mortality. Electrochemotherapy (ECT) combines chemotherapy with electroporation to enhance intracellular drug delivery, improving efficacy in various tumor models. In this study, we evaluated the effects of ECT using several novel anticancer drug candidates on MCF-7 breast cancer cells.

Methods: MCF-7 human breast adenocarcinoma cells were incubated with the novel drug candidates at various concentrations ranging from 0 to 200 uM, with or without electric pulse delivery. A standard electroporation protocol (eight 100-μs pulses at 1200 V/cm) was applied. Cell viability was assessed 24 hours post-treatment using MTT and Presto-Blue assays, YoPro-1 staining, and flow cytometry readouts to assess membrane permeabilization. Finally, the morphology of cells upon treatment with EP was assessed using holotomographic microscopy (HTM).

Results: Novel tested compounds induced a dose-dependent decrease in MCF-7 cell viability. We observed a U-shaped pattern in cells' permeability as the pulse frequency increased. Also, cell permeability increased with increasing electric field intensity up to the point of irreversible electroporation (>1600 V/cm). Combining with electroporation, new anticancer derivatives produced significantly higher cytotoxicity ($p < 0.05$ vs drug alone), with up to ~5-fold increase in cell death relative to drug alone. At the same time, holotomographic microscopy studies revealed the reversibility of cell membranes' distortions 30 min after pulse delivery.

Conclusion: These results demonstrate that ECT with novel drug candidates markedly enhances in vitro anticancer efficacy in MCF-7 cells. Electrochemotherapy may thus potentiate emerging chemotherapeutics, warranting further investigation towards clinical application in breast cancer.

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New 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide derivatives with electroporation-modulated activity in ovarian cancer

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Introduction: Ovarian cancer (OC) remains one of the gynecological malignancies with the poorest prognosis. Consequently, there is a continuous search for novel molecules with selective anticancer activity, as well as for physical methods capable of enhancing their cellular uptake. Electroporation (EP), through a transient increase in cell membrane permeability, facilitates the transport of poorly permeable compounds and constitutes the basis of electrochemotherapy (ECT). In ovarian cancer models, the combination of electric pulses with cisplatin, bleomycin, or calcium ions has been shown to significantly enhance cytotoxicity toward cancer cells, including those resistant to standard chemotherapy. Structures based on 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide have demonstrated promising anti-inflammatory, antioxidant, and anticancer activities, partially associated with COX-1/COX-2 inhibition and selective cytotoxicity toward malignant cells. Similarly, related pyridine cores and oligoamides exhibit activity against ovarian cancer cell lines, including cisplatin-resistant models, often with a favorable selectivity index. Against this background, the design of novel derivatives appears justified. The aim of this study was to evaluate their anticancer activity against OC cells and to determine whether this activity can be modulated and enhanced by electroporation, potentially combining the advantages of targeted chemotherapeutic strategies with physical enhancement of drug delivery.

Methods: Cytotoxicity of new 4,6-dimethyl-2-sulfanylpyridine-3-carboxamide derivatives was tested in SKOV-3 ovarian cancer cells using MTT. Cells were treated with various concentrations of the compounds with or without electroporation (EP). EP parameters were optimized by Yo-Pro-1 uptake (flow cytometry). Treatment-induced DNA damage was assessed using the comet assay. Changes in cells' morphology were observed using confocal microscopy.

Results: EP markedly increased Yo-Pro-1 uptake, confirming enhanced membrane permeability. Under these optimized conditions, a decrease in the viability of SKOV-3 cells treated with selected derivatives was observed compared to treatment with the compounds alone or EP alone. Moreover, the combination of selected derivatives with EP induced

significant DNA damage, as assessed by the comet assay, suggesting an enhanced cytotoxic effect mediated through genetic damage.

Conclusions: Our preliminary results suggest that the investigated 4,6 dimethyl 2 sulfanylpyridine 3 carboxamide derivatives may exhibit anticancer activity that can be further modulated by electroporation. However, additional experiments are required to confirm these findings and to fully elucidate the underlying mechanisms of action.

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