XIX

CONGRESS OF THE IBERIAN SOCIETY OF CYTOMETRY

Porto, Portugal

14.15.16
MAY
2025

VENUE

IPOPORTO

Content

01.
Welcome

Message from the Organization

Gabriela Martins
Chair Organizing Committee

WELCOME MESSAGE

Dear colleagues and friends,

It is with great pleasure that we invite you on behalf of the Board of the Iberian Society of Cytometry (SIC), the Organizing Committee and Scientific
Committee, to the XIX Congress of the Iberian Society of Cytometry, to be held from 14th to 16th may, 2025, in Porto (Portugal). We are greatly honoured to take on the responsibility that the SIC Board gave to us.

We will keep our programme with the educational and plenary sessions, conferences, symposia, providing the same opportunity for discussion.

We will expand the scope of our scientific program, with the participation of national and international expert speakers, as well as national and internacional research teams, through oral communications and posters.

Porto is a beautifull city of culture and knowledge and will be a perfect venue to host the Congress SIC 2025.

It is wonderful to meet you and to welcome you in this this charming city. We would like to thank you for your presence in the XIX SIC Congress, and we truly hope you enjoy attending it.

With warm regards,

02.
Committees

Organizing Committee
Scientific Committee

Organizing Committee

Gabriela Martins
Chair
Porto, Portugal

Jose-Carlos Segovia Sanz
Co-chair
Madrid, Spain

Julia Almeida
Co-chair
Salamanca, Spain

Bruno Fernandes
Vocals
Porto, Portugal

Carina Faria
Vocals
Porto, Portugal

Carla Azevedo
Vocals
Porto, Portugal

Carlos Palmeira
Vocals
Porto, Portugal

Catarina Fonseca
Vocals
Porto, Portugal

Maria Emília Sousa
Vocals
Porto, Portugal

Maria Inês Godinho
Vocals
Porto, Portugal

Scientific Committee

Alberto Orfão de Matos
Salamanca, Spain

Ana Lebre
Porto, Portugal

Artur Paiva
Coimbra, Portugal

Bruno Fernandes
Porto, Portugal

Carlos Palmeira
Porto, Portugal

Catarina Gregório Martins
Lisbon, Portugal

Cidália Pina Vaz
Porto, Portugal

Gabriela Martins
Porto, Portugal

Joana Caetano
Lisbon, Portugal

José Mário Mariz
Porto, Portugal

Jose-Carlos Segovia Sans
Madrid, Spain

José-Enrique O'Connor
València, Spain

Juana Ciudad
Salamanca, Spain

Julia Almeida
Salamanca, Spain

Lola Martinez Garcia
Madrid, Spain

Maria Arroz
Lisbon, Portugal

Rui Gardner
New York, USA

Rui Medeiros
Porto, Portugal

Tiago Guimarães
Porto, Portugal

03.
General Information

Information Map

GENERAL INFORMATION

Venue

Instituto Português Oncologia do Porto
Francisco Gentil, EPE - IPO Porto
Rua Dr. António Bernardino de Almeida 865,
4200-072 Porto (Portugal)
Tel: +351 225 084 000
Web: www.ipoporto.pt

Organized by

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28027 Madrid (Spain)
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Web: www.citometria.org
E-mail: secretaria.tecnica@citometria.org

Technical Support

Wednesday 14th April: 8.30h -19.00h
Thrusday 15th April: 8.30h -19.00h
Friday 16th April: 8.30h -14.00h
Tel: +351 914 592 380
Web: www.sic2025.pt
E-mail: geral@lab52.pt / eventos@lab52.pt

Congress Dadge

On arrival, at the registration desk you will receive your name badge. The organization kindly as you to carry always your badge with you at the Congress, during all sessions and social events.

Boat Trip

The boat trip will take place on the 15th, leaving from the Cais da Estiva, Ribeiro Porto (pier).
Time: 18:30h. Transfer by bus will be available from the venue. Please check the schedule at technical secretariat or at www.sic2025.pt

Closing Dinner

Closing dinner will be held on Thrusday 15th at VP Restaurant | WOW.
Rua do Choupelo, 39 4400-088 V.N. Gaia
Time: 20:00h. Bus transportation back to the venue will be available.Please check the schedule at technical secretariat or at www.sic2025.pt

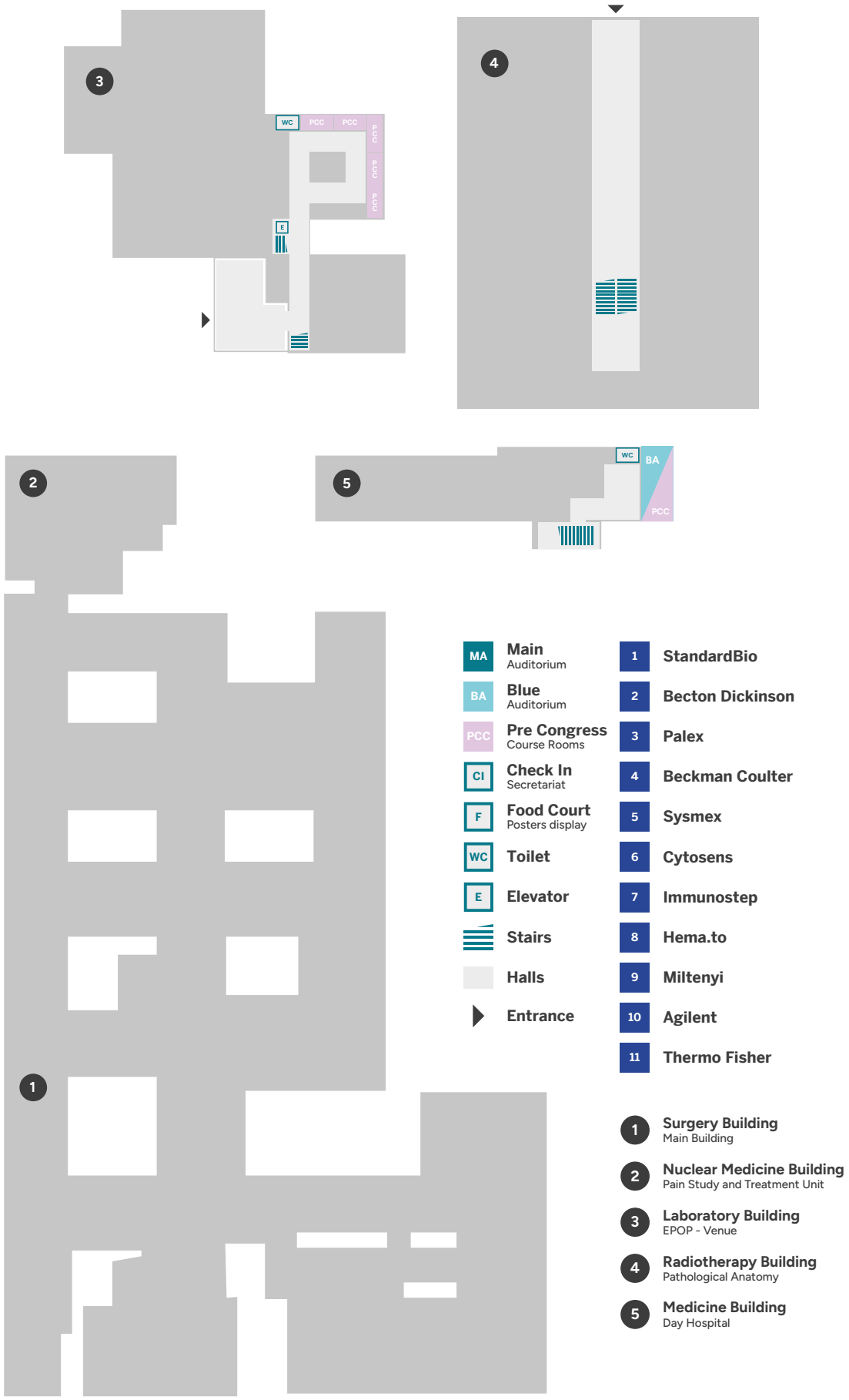
ORGANIZATION

IPOPORTO

SPC LABORATÓRIO DE IMUNOLOGIA
SETOR CITOMETRIA DE FLUXO

Sociedad Ibérica de Citometría

2ND FLOOR
FLOORPLAN



3RD FLOOR
FLOORPLAN

MA Main Auditorium	1 StandardBio
BA Blue Auditorium	2 Becton Dickinson
PCC Pre Congress Course Rooms	3 Palex
CI Check In Secretariat	4 Beckman Coulter
F Food Court Posters display	5 Sysmex
WC Toilet	6 Cytosens
E Elevator	7 Immunostep
Stairs	8 Hema.to
Halls	9 Miltenyi
Entrance	10 Agilent
	11 Thermo Fisher

1. **Surgery Building** — Main Building
2. **Nuclear Medicine Building** — Pain Study and Treatment Unit
3. **Laboratory Building** — EPOP - Venue
4. **Radiotherapy Building** — Pathological Anatomy
5. **Medicine Building** — Day Hospital

EXHIBITION
FLOORPLAN

03.
Programme

Short Version
14th June - Pre Congress Course & Congress
15th June - Congress
16th June - Congress

May 14th 2025

08:30 - 09:00	**CONGRESS OPENING & CHECK IN**
09:00 - 10:30	**PCC 01.** Part 1 **MEASURABLE RESIDUAL DISEASE IN MULTIPLE MYELOMA** **PCC 02.** Part 1 **B-CELL LYMPHOPROLIFERATIVE SYNDROMES** **PCC 03.** Part 1 **TIPS AND WARNINGS FOR YOUR FLOW CYTOMETRY EXPERIENCE** **PCC 04.** Part 1 **MEASURABLE RESIDUAL DISEASE IN ACUTE MYELOID LEUKAEMIA** **PCC 05.** Part 1 **HIGH-CONTENT FLOW CYTOMETRY DATA: FROM RESEARCH TO CLINICAL** Part 1
08:30 - 09:00	COFFEE BREAK
11:00 - 12:30	**PCC 01.** Part 2 **MEASURABLE RESIDUAL DISEASE IN MULTIPLE MYELOMA** **PCC 02.** Part 2 **B-CELL LYMPHOPROLIFERATIVE SYNDROMES** **PCC 03.** Part 2 **TIPS AND WARNINGS FOR YOUR FLOW CYTOMETRY EXPERIENCE** **PCC 04.** Part 2 **MEASURABLE RESIDUAL DISEASE IN ACUTE MYELOID LEUKAEMIA** **PCC 05.** Part 2 **HIGH-CONTENT FLOW CYTOMETRY DATA: FROM RESEARCH TO CLINICAL** Part 1
12:30 - 13:00	**REGISTRATION**
13:00 - 14:00	LUNCH
	MAIN AUDITORIUM
14:15 - 14:30	**WELCOME** Gabriela Martins, Julia Almeida, Júlio Oliveira, Jose-Carlos Segovia Sanz
14:30 - 15:30	OPENING CONFERENCE **MATHS FOR FLOW: THE EUROFLOW DATA ANALYSIS TOOLS** Chairs: Gabriela Martins, Julia Almeida, Jose-Carlos Segovia Sanz Alberto Orfão

	MAIN AUDITORIUM	BLUE AUDITORIUM
15:30 - 16:30	COMMERCIAL SESSION **THE WORLD'S FIRST SPECTRAL CELL ANALYZER WITH REAL-TIME IMAGING: BD FACSDISCOVER™ A8 CELL ANALYZER WITH BD CELLVIEW™ IMAGE TECHNOLOGY AND BD SPECTRALFX™ TECHNOLOGY** Mark Dessing, Rui Gardner	COMMERCIAL SESSION **INCORPORATION OF SPECTRAL CYTOMETRY INTO CLINICAL PRACTICE FOR THE DIAGNOSIS AND MONITORING OF ACUTE CHILDHOOD LEUKEMIA.** Manuel Ramírez Orellana **AURORA CS - CLINICAL APPLICATIONS IN THE CLINICAL PATHOLOGY DEPARTMENT IPO-PORTO** Bruno Fernandes
16:30 - 17:30	PARALLEL SESSION I **HEMATOLOGY** Advances in Diagnosis (Part 1) Chair: Bruno Fernandes, Gabriela Martins **Novel immunophenotypic approaches to improve diagnosis of mature T-cell neoplasms** Julia Almeida **Hodgkin's Lymphoma: The Use of Flow Cytometry in Diagnosis** Catarina Lau SHORT COMMUNICATION **Development and accreditation of spectral cytometry panels: Advances in the diagnosis and monitoring of leukocyte malignancy** Alfredo Minguela Puras	PARALLEL SESSION II **MICROBIOLOGY** Clinical and flow cytometry application Chair: Ana Lebre, José-Enrique O'Connor **Why use Flow Cytometry in the Fight Against Antimicrobial Resistance?** Cidália Pina Vaz **Determination of minimum inhibitory concentration of colistin directly from colonies, positive blood cultures and urine** Daniela Fonseca e Silva SHORT COMMUNICATION **Harnessing the Power of Lemon By-Products: Flow Cytometry Confirmation of Their Antibacterial Activity** Daniela Magalhães
17:30 - 18:00	COFFEE BREAK & INDUSTRIAL EXHIBITION	
18:00 - 19:00	PARALLEL SESSION I **HEMATOLOGY/IMMUNOLOGY** Advances in Diagnosis (Part 2) Chair: Maria Jorge Arroz, Artur Paiva **Inborn errors of immunity: revisiting the role of flow cytometry in the era of genetics** Catarina Gregório Martins **Using flow cytometry to uncover the microenvironment of acute myeloid leukemia** Delfim Duarte SHORT COMMUNICATION **Immunological biomarkers of CVID survival** Martin Perez-Andres	PARALLEL SESSION II **QUALITY CONTROL MANAGEMENT: ACCREDITATION AND CERTIFICATION** Quality and risk assessment in flow cytometry laboratories Chair: Tiago Guimarães, Juana Ciudad **Good practices in Cytometry research labs - the road to certification** Lola Martinez Garcia **Accreditation in Haematology Laboratories. Are we ready?** Enrique Colado

18:00 - 19:00	SHORT COMMUNICATION **Reproducibility of non-IVD-R certified spectral flow cytometers in multicenter settings: impact for applicability in collaborative clinical research** Silvia Martin	
	MAIN AUDITORIUM	
19:00 - 20:00	PLENARY SESSION **"25 YEARS OF QUALITY: THE IBERIAN PROGRAMME OF EQA (1999-2024)"** Martín Pérez-Andrés	
20:00 - 21:00	**WELCOME COCKTAIL**	

May 15th 2025

	MAIN AUDITORIUM	
08:30 - 09:30	PLENARY SESSION **FLOW CYTOMETRY ADVANCES IN PEDIATRIC SOLID TUMORS** Chairs: Bruno Fernandes, Ana Maia Ferreira Elaine Sobral	
	MAIN AUDITORIUM	**BLUE AUDITORIUM**
09:30 - 10:00	BECKMAN COULTER **CYTOFLEX MOSAIC: SPECTRAL FLOW CYTOMETRY MEETS MODULARITY** Marta Lopez	
10:00 - 10:30	BECKMAN COULTER **SUPERRCA - INTEGRATING FLOW CYTOMETRY WITH MOLECULAR BIOLOGY FOR THE ULTRA-SENSITIVE DETECTION OF RARE MUTATIONS** Sara Bodbin	SYSMEX **PRACTICAL DIAGNOSIS OF PNH, WHAT'S NEW IN 2025?** Enrique Colado
10:30 - 11:00	COFFEE BREAK & INDUSTRIAL EXHIBITION	
11:00 - 13:00	PARALLEL SESSION I **HEMATOLOGY** Minimal Measurable Disease Chairs: Sérgio Chacim, Ana Espírito Santo, Maria Dos Anjos Teixeira **Measurable Residual Disease in T-cell acute lymphoblastic leukemia** Łukasz Sędek **Measurable Residual Disease in Chronic Lymphocytic Leukemia** Joana Caetano	PARALLEL SESSION II **BIOTECHNOLOGY** Flow Cytometry in Human and Animal Research Chair: Carlos Palmeira , Jose-Carlos Segovia Sanz **Development of a customizable spectral flow cytometry mouse backbone panel for immune surveillance in normal and tumor tissues** Rui Gardner

11:00 - 13:00

Measurable residual disease in multiple myeloma
Bruno Paiva

SHORT COMMUNICATION
Detection of Circulating Tumor Plasma Cells after autologous stem cell transplantation in Multiple Myeloma patients: Predictive Value for Analytical Parameters Alterations and Disease Progression
Ana Rita Guedes

SHORT COMMUNICATION
Children ALL diagnose and follow-up according to ALLTogether protocol: IPO Porto experience
Bruno Fernandes

See what you sort with real-time imaging spectral flow cytometry
Òscar Fornas

Synergy for Precision Medicine: The Power of Combining Live-Cell Imaging with Acoustic Flow Cytometry
Jordi Petriz

The Good, the Bad and the Beautiful - A spectral cytometry journey
André Mozes

SHORT COMMUNICATION
Unlocking murine immune complexity: A standardized spectral flow cytometry toolbox for high-dimensional analysis
Lidia Silos

13:00 - 14:00 LUNCH & POSTERS VIEWING

14:00 - 16:00

MAIN AUDITORIUM

PARALLEL SESSION I
IMMUNOLOGY
Flow Cytometry in Non-Hematological Disease
Chairs: Maria Oliveira, Bruno Fernandes

Single-cell Proteome Profiling Reveals Distinct Immunological Patterns In The Lungs Of Patients With Acute Respiratory Distress Syndrome
Sebastiaan Joosten

Unravelling the human intestinal immunome in health and disease
David Bernardo Ordiz

Turning the glycocalyx into cancer vaccines: setting new paradigms for cancer immunotherapy
José Alexandre Ferreira

SHORT COMMUNICATION
The Immunosuppressive Landscape of Luminal B-like Breast Cancer Tumors
Tânia Moura

SHORT COMMUNICATION
Neutrophil APC-like cells: a distinct population of LDG with unique features and potential use as a biomarker of cardiovascular risk
Uxía Tobío-Parada

BLUE AUDITORIUM

PARALLEL SESSION II
BIOTECHNOLOGY
Functional FCM Applications
Chair: Enrique O'Connor, Carlos Palmeira

Flow Cytometric studies on modulation of platelet activation responses with clinical application
Maria do Céu Monteiro

Immunological and haemostasic studies in marine mammals
Alicia Martínez-Romero

SHORT COMMUNICATION
Comprehensive atlas of monocytic heterogeneity and differentiation across human tissues: a framework for clinical translation
Cristina Teodosio

16:00 - 16:30 COFFEE BREAK & INDUSTRY EXHIBITION

MAIN AUDITORIUM

16:30 - 17:30

PLENARY SESSION
SIC WORKING GROUPS & GECLID
Update of activities, user's meeting

SIC WORKING GROUPS
Grupo DHC: Ana Yeguas, Dolores Subirá
Grupo de Trabajo Acreditación en laboratorios de Citometría: Catarina Martins, Juana Ciudad

GECLID User's Annual Assembly: new proposals in cell immunity QA
Carmen Martín Alonso

May 16th 2025

	MAIN AUDITORIUM	BLUE AUDITORIUM
09:00 - 11:00	PARALLEL SESSION I **IMMUNOLOGY** CAR-T Immunotherapy: An Update Chairs: Alberto Orfão, José Mário Mariz **The academic development of CART-cells** Julio Delgado **Role of Flow Cytometry in CAR-T cell manufacturing and quality control** Estefanía Garcia Guerrero **Advancing CAR-based immunotherapy for cancer** Pablo Menéndez SHORT COMMUNICATION **Functional Characterization of CAR-T Cell Activation, Exhaustion and Cytokine Production** Ernesto Marcos Lopez SHORT COMMUNICATION **Development of an innovative reagent for a standarized anti-CD19 CAR-T cells detection by Flow Cytometry** Eduarda Da Silva Barbosa	PARALLEL SESSION II **BIOTECHNOLOGY** Flow Cytometry and Extracelular Vesicles: What´s new Chairs: Helena Vasconcelos, André Mozes **Standardization of extracellular vesicle measurements by flow cytometry** Britta A. Bettin **Characterization and isolation of Extracellular Vesicles by Flow Cytometry** Òscar Fornas **R-EV-eling in the new toys and complements** Alfonso Blanco

11:00 - 11:30 COFFEE BREAK & INDUSTRIAL EXHIBITION

MAIN AUDITORIUM

11:30 - 12:00

SIC GENERAL ASSEMBLY
Chairs: SIC Board

12:00 - 12:30

SIC AWARDS CEREMONY
Chairs: Julia Almeida, Jose-Carlos Segovia Sanz

12:30 - 13:15	CLOSING LECTURE **REGULATORY T CELLS FOR THE TREATMENT OF CHRONIC GRAFT VERSUS HOST DISEASE** Chairs: Julia Almeida, Jose-Carlos Segovia Sanz João Forjaz de Lacerda
13:15 - 13:30	**CLOSING CEREMONY** Chairs: Julia Almeida, Jose-Carlos Segovia Sanz, Gabriela Martins, Carlos Palmeria, Bruno Fernandes

May 14th
Pre Congress Course

PCC 01. Measurable residual disease in multiple myeloma
PCC 02. B-Cell lymphoproliferative syndromes
PCC 03. Tips and warnings for your flow cytometry experience
PCC 04. Measurable residual disease in acute myeloid leukaemia
PCC 05. High-content flow cytometry data: From research to clinical

Pre-Congress Course 01.

Measurable residual disease in multiple myeloma

Practical Case Analysis

Juan Flores Montero
Coordinator

Salamanca, Spain

Attendees	Duration	Room
20	180 min.	2 EPOP

Target Audience

Intermediate / Advanced cytometrist with working knowledge on MM MRD detection

Description

Fully practical, hands-on session on analysis of BM MRD cases from MM patients after treatment, using both classic and advanced (i.e., data base-based) analysis strategies and the Infinicyt software in an interactive atmosphere, favoring discussion aiming at dealing with common (and less common) challenges. The complete process from NGF analysis to reporting will be reviewed. Bring along your questions to enrich the interaction. We encourage active participation to make the most of this course

Learning Objectives

After participating in the course, participants will:

- Understand pre-analysis quality of sample checks to guarantee accurate results
- Follow a comprehensive classical (manual) analysis strategy for this application
- Gain experience of automated (software powered) analysis
- Overcome common hurdles of MM MRD data analysis
- Identify the minimally required information to be reflected during reporting

Pre-Congress Course 02.

B-Cell lymphoproliferative syndromes

Practical Case Analysis

Maria Arroz
Coordinator

Lisbon, Portugal

Attendees	Duration	Room
20	180 min.	3 EPOP

Target Audience

Intermediate level – Participants should be already experient in the analysis of case files.

Description

Overview of Chronic B-cell lymphoproliferative disorders (B-CLPD); supervised hands-on group analysis of distinct diseases in different types of samples using the Infinicyt™ software. Interactive discussion is encouraged.

Learning Objectives

By the end of this course the participants should be able to:

1. Distinguish reactive from clonal proliferations of mature B-cells, both at diagnosis and during follow-up.
2. Identify key features of the most frequent pathologies.

Pre-Congress Course 03.

Tips and warnings for your flow cytometry experience

Mariana Rafael Fernandes
Coordinator

Lisbon, Portugal

Ana Helena Santos
Coordinator

Porto, Portugal

Andreia Miranda
Coordinator

Porto, Portugal

Catarina Meireles
Coordinator

Porto, Portugal

Attendees	Duration	Room
20	180 min.	Blue Auditorium

Target Audience

Scientific Community, such as clinicians, technicians, students and researchers interested in advancing flow cytometry expertise for diagnostics and translational research.

Description

Deepen your understanding of flow cytometry with this workshop that covers key suggestions and pitfalls to avoid for polychromatic, spectral, and imaging cytometry.

This workshop can be an asset for those who are just starting to plan flow cytometry experiments, but also for more experienced participants. It will cover various topics to take into account when planning flow cytometry experiments, as well as some practical tips to ensure their success. There will also be an opportunity for clinicians and researchers to get together to implement and share knowledge and good practices in Flow Cytometry.

Successful flow cytometry analysis requires a single-cell suspension, careful management of pre-analytical and analytical variables in sample preparation, and strategies to troubleshoot technical challenges and improve data quality for research and clinical applications.

Learning Objectives

- Understanding the Fundamentals of Flow Cytometry Workflow
- Overview of Polychromatic, Spectral, and Imaging Flow Cytometry instruments
- Experimental Planning
- Sample Preparation tips & tricks
- Tissue Sample Processing in Research and Diagnosis
- Panel Design
- Controls and Standardization
- Troubleshooting and Optimization
- Bridging Research and Clinical Applications

Pre-Congress Course 04.

Measurable residual disease in acute myeloid leukaemia

Practical Case Analysis

Sergio Matarraz
Coordinator

Salamanca, Spain

Attendees	Duration	Room
20	180 min.	4 EPOP

Target Audience

Flow cytometry specialists

Description

Participants will gain a pragmatic understanding of normal and leukemic myeloid differentiation patterns (based on more conventional and new informative markers), equipping them to identify aberrant cells and flow cytometry patterns indicative of distinct AML diagnostic entities. This skill is critical for the monitoring of measurable residual disease (MRD) using flow cytometry.

Learning Objectives

1. Identification of major differentiation trajectories in normal bone marrow (BM) and peripheral blood (PB) CD34+ hematopoietic progenitor and precursor cells (HPC).
2. Highlight the contribution of lineage infidelity markers and other leukemia associated phenotypes in the diagnosis of distinct AML entities for integration in MRD panels. Relevance of using NGF databases.
3. Outline an alternative strategy to characterize CD34+ HPC in BM and PB along AML treatment, independent of baseline diagnostic data.

Pre-Congress Course 05.

High-content flow cytometry data: from research to clinical

Mattia Gallizioli
Coordinator

Barcelona, Spain

Javier Silván
Coordinator

Madrid, Spain

João Moura Alves
Coordinator

Munich, Germany

Attendees	Duration	Room
200	180 min.	Main Auditorium

Target Audience

Researchers and clinicians, regardless of their level of experience, confronted with the need to analyze and interpret a rapidly growing and increasingly complex volume of flow cytometry data

Description

High quantitative analysis of flow cytometry data allows for precise characterization and measurement of cellular populations, cell functions, and molecular markers, which are crucial in different fields from research to clinical. This analysis should be performed by specialized software that provides advanced features for data visualization, analysis, and interpretation, ensuring accurate and efficient analysis of multi-parameter flow cytometry data. In the present course will be discussed three analysis programs (FlowJo, Infinicyt and Hema.to) and their applications and innovative strategies for research and clinical cell analysis. Can these different tools be used in an integrated and complementary way?

Learning Objectives

At the end of the course, participants should:

- Gain a clear understanding of how each analysis program can enhance their work;
- Identify which program is best suited for their specific data and experimental context;
- Familiarize themselves with the fundamental principles and functionalities of each program;
- Additionally, they should contact with the latest modules and tools available for more advanced analyses and specialized applications.

May 14th
Congress

OPENING CONFERENCE
MATHS FOR FLOW: THE EUROFLOW DATA ANALYSIS TOOLS

COMMERCIAL SESSION - BECTON DICKINSON
THE WORLD'S FIRST SPECTRAL CELL ANALYZER WITH REAL-TIME IMAGING: BD FACSDISCOVER™ A8 CELL ANALYZER WITH BD CELLVIEW™ IMAGE TECHNOLOGY AND BD SPECTRALFX™ TECHNOLOGY

COMMERCIAL SESSION - PALEX
INCORPORATION OF SPECTRAL CYTOMETRY INTO CLINICAL PRACTICE FOR THE DIAGNOSIS AND MONITORING OF ACUTE CHILDHOOD LEUKEMIA / AURORA CS - CLINICAL APPLICATIONS IN THE CLINICAL PATHOLOGY DEPARTMENT IPO-PORTO

PARALLEL SESSION I
HEMATOLOGY
Advances in Diagnosis (Part 1)

PARALLEL SESSION II
MICROBIOLOGY
Clinical and flow cytometry application

PARALLEL SESSION I
HEMATOLOGY/IMMUNOLOGY
Advances in Diagnosis (Part 2)

PARALLEL SESSION II
QUALITY CONTROL MANAGEMENT: ACCREDITATION AND CERTIFICATION
Quality and risk assessment in flow cytometry laboratories

PLENARY SESSION
"25 YEARS OF QUALITY: THE IBERIAN PROGRAMME OF EQA (1999-2024)"

OPENING CONFERENCE

MAIN AUDITORIUM

MATHS FOR FLOW: THE EUROFLOW DATA ANALYSIS TOOLS

Chair: Gabriela Martins, Julia Almeida, Jose-Carlos Segovia Sanz

Alberto Orfão
Salamanca, Spain

When starting a flow cytometry laboratory, the availability of appropriate instrumentation and reagent panels that cover the envisaged applications of use, frequently emerge as the most relevant components to assure they are in place in both research and clinical laboratories. In contrast, appropriate software programs for data analysis are typically considered a secondary tool integrated into the flow cytometer instrument, whose functionalities are standard and not critically relevant. Thus, a software should generally support data acquisition, fluorescence compensation and gating; in addition, it includes basic descriptive statistics and preferably, colourful graphical representations of bivariate dot plots and single parameter histograms for comprehensive expert-guided gating and identification of cell populations and subsequent data interpretation.

In the last decades the volume of flow cytometry data per file has exponentially increased due to the measurement of greater numbers of cells stained with a still increasing number of markers that allow simultaneous identification of hundreds to thousands of different cell populations in a single sample. These new features of flow cytometry data files make the use of conventional gating procedures extremely laborious and not feasible anymore even whenever the speed and memory capacities of computers have increased exponentially. In turn, such increased power of computer hardware has facilitated important changes in flow cytometry software.

EuroFlow has pioneered these changes since 2006 through the incorporation of new mathematical algorithms into software tools to 1) accelerate the design and optimization of antibody panels, 2) automatically identify those cell populations that coexist in a sample, and 3) by providing new tools for the biological and clinical interpretation of the data collected. Thus, flow cytometrists became familiarized with new terms such as clustering analysis, multivariate principal component analysis (PCA), canonical regression analysis, support-vector machine (SVM), nearest-neighbour, neural networks, data calculation and normalization. In parallel, new graphical plots and their abbreviations, such as the automated population separator (APS), canonical analysis (CA), T-distributed stochastic neighbour embedding (t-SNE), uniform manifold approximation and projection (UMAP) plots have been introduced in routine flow cytometric analyses. Those innovative software tools have set the basis for an expert-guided semi- and fully automated data analysis, interpretation and reporting of flow cytometry data and results. Altogether, these new software programs contribute to faster, easier and better controlled flow cytometry data analysis. Moreover, they provide the basis for an improved understanding and extraction of the information contained in a flow cytometry data file, and to its application in both the research and clinical diagnostic fields.

Thus, the development of new software tools based on the incorporation of relatively “old” mathematic algorithms, and particularly on combinations of such mathematic algorithms, specifically translated for use with flow cytometry data, has exploded in recent years and made software one of the most important components of a flow cytometry laboratory.

COMMERCIAL SESSION

MAIN AUDITORIUM

Mark Dessing
Almere, Netherlands

THE WORLD’S FIRST SPECTRAL CELL ANALYZER WITH REAL-TIME IMAGING: BD FACSDISCOVER™ A8 CELL ANALYZER WITH BD CELLVIEW™ IMAGE TECHNOLOGY AND BD SPECTRALFX™ TECHNOLOGY

Imagine if you could analyze cells with spectral flow cytometry and access single-cell images all at once. No need for separate experiments. No need for additional instrumentation and no need for complicated software and workflows. This possibility will soon become a reality with the new BD FACSDiscover™ A8 Cell Analyzer. This instrument enables high-parameter experiments reducing variability by confirming the cells you’re analyzing are the same cells you are viewing through live visualization. You may even discover new insights that were previously undetectable.

Rui Gardner
New York, USA

During the first part of the talk, Mark Dessing will guide you through the integrated BD SpectralFX™ Technology, enabling high-parameter spectral workflows, expanded panel size and increased flexibility with 78 fluorescent detectors across five lasers, together with BD CellView™ Image Technology, enabling cell image acquisition so that imaging data can be quantified in real-time. Adding single-cell imaging to a flow cytometry workflow helps address questions that couldn't be previously answered by flow cytometry including spatial and physical characteristics of cells such as morphology and protein localization.

This will be followed by the opportunity to see some of the exciting applications Rui Gardner explored while working with a BD FACSDiscover™ A8 prototype.

COMMERCIAL SESSION

BLUE AUDITORIUM

Manuel Ramírez Orellana
Madrid, Spain

INCORPORATION OF SPECTRAL CYTOMETRY INTO CLINICAL PRACTICE FOR THE DIAGNOSIS AND MONITORING OF ACUTE CHILDHOOD LEUKEMIA.

Palex

Conventional flow cytometry has been used for decades to identify, quantify, and monitor the presence of cell populations defined by the expression profile of specific markers. Specifically, in the field of hematological malignancies in children and adolescents, flow cytometry allows for the identification of the type and lineage of each patient's disease and also sensitively quantifies the presence of disease at precise time points during treatment, i.e., the levels of measurable residual disease (MRD). Quantifying MRD using flow cytometry has allowed for the adaptation of personalized therapies to each patient, contributing to improved outcomes for various protocols in recent decades.

Spectral flow cytometry has been used in experimental laboratories for research purposes for quite some time. However, the clinical diagnostic environment differs considerably from that of a research laboratory in many aspects: turnaround times, workflows, pre-analytical phase, the requirement to follow standardized work protocols, and reagent use, among others, which often increases complexity (at least initially) and cost. Validation studies are necessary before transferring spectral flow cytometry from the laboratory to clinical practice, allowing it to be incorporated into the daily routine of a diagnostic laboratory. We present a comparative study of both technologies, spectral flow cytometry versus conventional flow cytometry, in primary samples corresponding to the diagnosis and follow-up of children and adolescents with acute lymphoblastic leukemia.

Bruno Fernandes
Porto, Portugal

AURORA CS - CLINICAL APPLICATIONS IN THE CLINICAL PATHOLOGY DEPARTMENT IPO-PORTO

The Aurora CS provides the benefits of Full Spectrum Profiling technology combined with the flexibility required to meet various biological and sorting conditions that can be used for research or clinical purposes.

In this brief presentation will be discussed some clinical applications for this technology, highlighting some practical insights and experience from the IPO Porto Clinical Pathology Service.

HEMATOLOGY

Advances in Diagnosis (Part 1)

Chair: Bruno Fernandes, Gabriela Martins

Julia Almeida
Salamanca, Spain

NOVEL IMMUNOPHENOTYPIC APPROACHES TO IMPROVE DIAGNOSIS OF MATURE T-CELL NEOPLASMS

T-cell chronic lymphoproliferative disorders (T-CLPD) are a group of rare malignant diseases derived from mature T lymphocytes, comprising numerous tumors highly heterogeneous in their clinical presentation, phenotypic/morphologic features, pathogenesis and prognosis. Currently, the diagnosis (including diagnosis of clonality) of these neoplasms and their subsequent classification into precise diagnostic categories recognized by international systems of classification (i.e., WHO and ICC) is still a challenge. The EuroFlow Consortium has innovated flow-cytometry applications for the diagnosis and classification of hematological malignancies and generated reproducible results across individual laboratories, and these strategies have been also applied to T-CLPD. Accordingly, sequential steps including the application of a screening tube (Lymphocyte Screening Tube, LST), diagnosis of T-cell clonality and finally classification into precise WHO/ICC categories using EuroFlow FCM panels and protocols have been developed. We confirmed that LST is a useful approach to identify the precise T-cell subtype involved in the lymphocytosis, and even it allows the identification of T-cell associated aberrancies in more than 90% of cases. As regards diagnosis of T-cell clonality, new strategies recently emerged (and will be presented) to overcome the classical limitation of an absence of a rapid and reproducible assay to easily assess T-cell clonality by flow cytometry, thanks to the development, validation and implementation of flow-cytometry based approaches, based on the use of antibodies specific for the mutually exclusive TRBC1 and TRBC2 isoforms. Finally, we developed novel tools based on immunophenotyping, aimed at the automated classification of the most common leukemic T-CLPD (T-prolymphocytic leukemia, Sézary syndrome/mycosis fungoides and T-large granular lymphocytic leukemia), and preliminary results will be presented. Further, we classified tumor cells from T-CLPD into precise functional- and maturation-associated compartments, based on their phenotypic similarities with their normal maturation-related and functional-associated T-cell counterparts, confirming the presence of distinct phenotypic patterns resembling specific maturation associated and Th-related profiles of normal T-cells among distinct diagnostic categories of T-CLPD, which might contribute to a more precise classification of T-CLPD. In summary, EuroFlow standardized flow-cytometry approaches highly contribute to improve the diagnosis and classification of T-CLPD.

Catarina Lau
Porto, Portugal

HODGKIN'S LYMPHOMA: THE USE OF FLOW CYTOMETRY IN DIAGNOSIS

Hodgkin lymphoma (HL) encompasses a heterogeneous group of B-cell-derived lymphoid neoplasms, classically divided into two major entities: classical Hodgkin lymphoma (cHL), which accounts for the majority of cases (90–95%), and nodular lymphocyte predominant Hodgkin lymphoma (NLPHL), representing a biologically and clinically distinct minority (5–10%). In the latest International Consensus Classification (2022), NLPHL has been redefined as nodular lymphocyte predominant B-cell lymphoma (NLPBL) to reflect its biological and clinical proximity to other indolent B-cell lymphomas. However, the WHO 5th edition (2022) continues to classify it within the Hodgkin lymphoma group, underscoring ongoing debates in lymphoma taxonomy.

Classical Hodgkin lymphoma comprises four histological subtypes—nodular sclerosis, mixed cellularity, lymphocyte-rich, and lymphocyte-depleted—each exhibiting distinct immunophenotypic and clinical features. Despite recent advances in understanding the pathogenesis and microenvironment of these entities, the diagnosis of HL remains challenging, primarily due to the sparse distribution of neoplastic cells within a heterogeneous inflammatory background, which can mimic reactive conditions or other lymphoproliferative disorders.

Traditionally, histopathology and immunohistochemistry (IHC) have been the mainstays of HL diagnosis. However, flow cytometry (FCM)—previously considered of limited utility in HL due to the rarity and fragility of HRS cells—has gained traction as a valuable adjunct, particularly in cHL. Several studies have demonstrated that FCM can detect HRS cells using specific antigen panels (e.g., CD30, CD15, CD40, CD95), or more commonly, identify distinct immunophenotypic signatures in the surrounding immune cell populations.

This presentation will review the updated classification of HL, outline the diagnostic difficulties posed by the inflammatory milieu and scarcity of neoplastic cells, and explore the evolving utility of flow cytometry. We will discuss both the technical and clinical aspects of implementing FCM in HL diagnosis and share our institutional experience, including representative case studies that illustrate how flow cytometry complements traditional methods and enhances diagnostic precision. By bridging histopathological and immunophenotypic data, flow cytometry stands as a promising tool in the diagnostic approach to Hodgkin lymphoma.

PARALLEL SESSION II

BLUE AUDITORIUM

MICROBIOLOGY

Clinical and flow cytometry application

Chair: Ana Lebre, José-Enrique O'Connor

Cidália Pina Vaz
Porto, Portugal

WHY USE FLOW CYTOMETRY IN THE FIGHT AGAINST ANTIMICROBIAL RESISTANCE?

Antimicrobial resistance (AMR) is a silent pandemic, responsible for more deaths worldwide than cancer or HIV. Several factors contribute to AMR, including the use of antibiotics in agriculture, veterinary medicine, and human healthcare. However, diagnostic delays also play a significant role. Traditional susceptibility testing (AST) in clinical laboratories relies on the growth of microorganisms in the presence of antimicrobials, which typically takes 2–3 days to yield results.

Critically ill patients often require immediate and effective treatment, yet empirical therapy fails in up to 40% of cases, contributing to increased morbidity and mortality. While molecular methods have accelerated microbial identification, they offer limited insight into antimicrobial susceptibility, as resistance mechanisms are not always predictable from genomic data alone.

Phenotypic testing remains essential, but time is of the essence. Flow cytometry allows us to assess cellular responses to antimicrobial exposure in as little as one hour. By combining this with a multiparametric algorithm powered by artificial intelligence, we can classify bacterial strains as susceptible, resistant, or intermediate within a maximum of two hours.

Our work began with testing pure colonies, then expanded to positive blood cultures, and more recently to urine samples screened positive by flow cytometry. A CE-IVD product is now in the market and being used by hospitals. We believe the transformative impact that flow cytometry had in haematology and cytology is now reaching microbiology. This approach has the potential to not only improve outcomes for infected patients but also promote more rational antibiotic use and help curb the spread of AMR.

Daniela Fonseca e Silva
Porto, Portugal

DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION OF COLISTIN DIRECTLY FROM COLONIES, POSITIVE BLOOD CULTURES AND URINE

The emergence of multidrug-resistant (MDR) Gram-negative bacteria is a growing problem. Colistin is used as a "last-line" therapy to treat this infections, when essentially no other options are available.

EUCAST and CLSI do not recommend disk diffusion methods, Etest for minimum inhibitory concentration (MIC) determination. Microdilution is time-consuming.

We used Flow cytometry to evaluate antimicrobial susceptibility and it seems to be an excellent tool for evaluation of colistin susceptibility directly from colonies, blood culture and urine samples, with high agreement with broth microdilution reference method, providing a MIC.

This methodology is easier to perform in clinical labs and providing results in useful clinical time, being an alternative protocol.

HEMATOLOGY/IMMUNOLOGY

Advances in Diagnosis (Part 2)

Chair: Maria Jorge Arroz, Artur Paiva

Catarina Gregório Martins
Lisboa, Portugal

INBORN ERRORS OF IMMUNITY: REVISITING THE ROLE OF FLOW CYTOMETRY IN THE ERA OF GENETICS

Over the past decades, the field of primary immunodeficiency evolved significantly, not only in terminology and classification but also through the development of emerging diagnostic and therapeutic approaches. Notably, these disorders are now referred to as Inborn Errors of Immunity (IEI), a designation that better captures the wide spectrum of clinical and genetic features encompassed by the ten groups currently recognized, ranging from the paradigmatic Severe Combined Immunodeficiencies (SCID) to the expanding collection of IEI phenocopies.

Furthermore, the diagnostic landscape of IEI is also rapidly changing, driven by groundbreaking advances in genetic diagnostics, increasingly available worldwide. Indeed, targeted gene panels, whole exome (WES) and whole genome sequencing (WGS), RNA sequencing, and proteomics approaches are transforming patient evaluation but also our broader understanding and classification of IEI. The 2024 update of the International Union of Immunological Societies (IUIS), published in April 2025, now lists 559 distinct IEI, including 67 newly identified monogenic defects, almost double the numbers reported a decade ago.

Historically, flow cytometry has established itself as a cornerstone in the diagnosis, screening, and monitoring of IEI patients, offering a versatile spectrum of assays, from basic immunophenotyping to high-dimensional profiling of lymphoid and non-lymphoid compartments. Now, as the novel genetic insights become pivotal in clinical immunology and patient care, flow cytometry is adapting and assuring its role as complementary and essential partner in the diagnostic workflow, to enable early and precise diagnoses, personalized therapeutic strategies, and critical information for genetic counseling.

For instance, while genomic sequencing can reveal causative variants, interpretation often depends on functional validation, a domain where flow cytometry continues to excel. It remains indispensable for assessing immune cell subset distributions, receptor expression, intracellular signaling, and functional displays, like cellular activation or apoptosis, thereby helping to determine the classification and pathogenicity of variants of uncertain significance (VUS). Importantly, flow cytometry can also guide genetic workflows, providing immunophenotypic clues that help prioritize gene targets, ultimately reducing diagnostic turnaround times. Also, where genetic testing is not universally accessible or not an affordable alternative, flow cytometry remains the most rapid and cost-effective diagnostic tool for timely diagnostic and therapeutic decision-making. Thus, rather than overshadowed by genomic technologies, flow cytometry is advancing in parallel, reaffirming its critical role as a front-line tool in the modern IEI diagnostic algorithms.

A highly practical example of this integrated approach is newborn screening for SCID, which offers a transformative opportunity for early diagnosis and curative treatment of life-threatening IEIs. In Portugal, as in many other European and non-European countries, pilot implementation projects are underway using quantification of T-cell receptor excision circles (TRECs) via real-time quantitative PCR (qPCR) as initial screening strategy. Infants with absent or low TREC levels are flagged for follow-up: beyond the standard complete blood count approach, immunophenotyping of T, B, and NK cells, with extended characterization of naïve and memory phenotypes, provide essential insights into the nature of the immune defect. Testing for T cell function, often using flow cytometry, or determining maternal T cell engraftment, further refine the immunological assessment, and in turn, guide the subsequent targeted or broad-based genetic testing. This coordinated diagnostic cascade exemplifies how flow cytometry remains central, not only for initial evaluation, but also for contextualizing and validating genetic findings.

More than ever before, though, these major advances, allied with the growing demand for addressing secondary causes of immunodeficiency, highlight the urgent need for standardized strategies in both immunophenotyping and functional assays. The continued use of heterogeneous panels or limited access to functional testing can significantly restrict the diagnostic potential of laboratories, often facing the dual challenge of managing rare conditions with broad clinical heterogeneity and responding to an increasing range of immune modulating therapies. A harmonized, robust, and standardized approach is essential for diagnostic accuracy but also for monitoring immune reconstitution and evaluating therapeutic responses across diverse clinical contexts.

In this new era, marked as well by the rise of artificial intelligence, flow cytometry not only endures; it flourishes as a vital, ever-evolving interface between immunology and genomics, guiding a more integrated and precision-focused path for the diagnosis and care of patients with IEI.

Delfim Duarte
Porto, Portugal

USING FLOW CYTOMETRY TO UNCOVER THE MICROENVIRONMENT OF ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is an aggressive leukemia characterized by rapid proliferation of malignant myeloid blasts in the bone marrow (BM). Overall survival remains 60 years old. Persistence of measurable residual disease (MRD) after chemotherapy identifies patients at risk for disease relapse. It has been shown that AML cells interact with different components of the BM microenvironment to promote disease progression and chemoresistance. However, a comprehensive analysis of this interdependence during the several stages of disease is still lacking.

We modified clinically relevant mouse models of AML to express a novel niche labelling system and characterized the niche interactome of AML at disease progression and at MRD persistence. We uncovered the role of non-classical monocytes with anti-leukemic activity in AML and studied the endothelial niche in leukemia. We also found that erythroblastic islands are actively remodeled in AML, leading to changes in systemic iron levels. These findings, contribute to the understanding of AML disease mechanisms and to the development of new therapies, such as iron manipulation in AML.

PARALLEL SESSION II

BLUE AUDITORIUM

QUALITY CONTROL MANAGEMENT: ACCREDITATION AND CERTIFICATION

Quality and risk assessment in flow cytometry laboratories

Chair: Tiago Guimarães, Juana Ciudad

Lola Martinez Garcia
Madrid, Spain

GOOD PRACTICES IN CYTOMETRY RESEARCH LABS - THE ROAD TO CERTIFICATION

Currently certification is not mandatory for research cytometry labs but even if it is not the objective to get a certification from an external body, but following good practices in the core facility is a good way to ensure the best outcome from your users and research projects. We will discuss which areas should be covered to ensure good practices are followed in your lab, including:

- Equipment management and validation
- Standard operating procedures (SOPs)
- Quality assurance and controls
- Personnel training and competency
- Biosafety
- Data management

Many of these steps are common and need to be fulfil if you wish to get the ISO 17025 norm or simply engage with the recognition program from the International Society for the Advancement of Cytometry (ISAC).

Enrique Colado
Oviedo, Spain

ACCREDITATION IN HAEMATOLOGY LABORATORIES. ARE WE READY?

Accreditation is “a third-party recognition of competence to perform specific tasks” which attests that a laboratory has been successful in meeting the requirements of international accreditation standards. For medical laboratories, confirms the successful implementation of elements of a comprehensive quality management system according to ISO 15189:2022. While some laboratories have experience with previous ISO 15189 iterations, others may be now considering to pursue accreditation for a single or several tests.

In this session we will go through the most important items when considering applying for accreditation on a clinical flow cytometry laboratory. This goes beyond good laboratory practices, certification standards or quality

standards, as accreditation aims to demonstrate technical competence (methods are selected, developed, modified, validated and verified in proper applications), responds to requirements for patient care and the laboratory is fully competent in the specific method. Currently, the introduction of accreditation is uneven within our European environment. The number of countries where the health authorities require clinical laboratory accreditation either for all of their activities or for some disciplines or specific tests is increasing. Challenges as the implementation of the European in vitro Diagnostic and in vitro diagnostics medical devices Regulations (IVDR) (EU) 2017/745 and (EU) 2017/746 will force our laboratories to navigate through a transition period in which uncertainty of what and when should be done.

In the view of accreditation bodies, clinical flow cytometry is usually placed within an accredited clinical sector, for example in immunology or haematology. Accreditation bodies as Instituto Português de Acreditação (IPAC) or Entidad Nacional de Acrediation (ENAC), in Portugal and Spain respectively, engage in international activities (participating in EA (European Accreditation) and ILAC (International Laboratory Accreditation Cooperation) working groups to ensure homogeneity among accreditation bodies, therefore, accredited tests in Portugal and Spain have worldwide recognition. Of note, the Iberian Society of Cytometry (SIC) has signed an agreement with ENAC to increase the knowledge on every part of the accreditation process, including laboratories, auditors and users. Apart from providing a longstanding External Quality Assurance program, the SIC has nurtured the creation of an Accreditation Working Group within the society. The main objective of this Working Group is to promote and facilitate the continuous training of SIC members on issues related to laboratory accreditation, with a special focus on clinical flow cytometry, and to improve collaboration with the accreditation bodies, helping to define quality recommendations applicable to cytometry laboratories, based on the exchange of experiences, doubts and specific adaptations of the accreditation standards. For example, former Management criteria are not different from any medical laboratory, but they have expanded taking special interest in risk management. Although these criteria may seem difficult to comply in smaller laboratories lacking adequate support from quality units, they can be achieved. Specific flow cytometry standards or technical criteria are not included, which may be problematic as so clinical cytometrists may find themselves lacking guidance because a significant number of the methods are laboratory developed tests (LDTs)/In-house tests. Several organizations such as the European Clinical Cytometry Society, the International Council for Standardization of Haematology and International Clinical Cytometry Society or the Clinical Laboratory Standards Institute have provided guidelines for the general validation of cytometry assays. On the other hand, several working groups or consortia provide clear guidelines on specific diseases. Other additional resources are technical notes both from accreditation bodies and societies, which greatly clarify the requirements for quality and competence and also identify major areas of variability, determine critical components needing standardization develop and define acceptability standards and criteria, and provide guidance and measures for practical implementation in the laboratory. These guidelines and technical notes are widely applicable from larger to smaller laboratories.

Regarding the question of whether our laboratories are ready for accreditation, I am largely optimistic. Many laboratories have successfully accredited one or several tests and many more have shown competence in key aspects of the requirements for accreditation. For the latter, individualised planning to implement the requirements is necessary. Accreditation is more an instrument than the aim that increases the quality of clinical services. Accreditation is not about who the best is, but who has a system of standard procedures which are safe, confidential and professional.

PLENARY SESSION

MAIN AUDITORIUM

"25 YEARS OF QUALITY: THE IBERIAN PROGRAMME OF EQA (1999-2024)"

Martín Pérez-Andrés
Salamanca, Spain

Martin Perez-Andres (1,2); Alba Torres-Valle (1); José Alcaraz (3,4); Oscar Redondo (1,2); Marta Palomo (3); Alberto Orfao (1,2)

1. Service of Cytometry &Department of Medicine, CICancer-University of Salamanca, Salamanca, Spain
2. External Quality Control Scheme of the Iberian Society of Cytometry (SIC)
3. Laboratorio de Evaluación Externa de la Calidad en Hematología, Hospital Clinic de Barcelona, Barcelona, Spain
4. External Quality Control Scheme of the Sociedad Española de Hematología (SEHH)

The Iberian Society of Cytometry External Quality Assessment Programme started in 1999 and provides external quality assessment (EQA) to laboratories from Spain, Portugal and Latin America affiliated to the Iberian Society of Cytometry (Sociedad Ibérica de Citometría SIC) and, since 2021, to the Spanish Hematology Society (Sociedad

Española de Hematología) in collaboration with the University of Salamanca (USAL) and the Laboratory for External Quality Assessment (LEECH). In 2024, more than 70 clinical laboratories participated in at least one of the seven EQA programmes: Immunophenotyping of Leukaemia and Lymphoma (1999-), Analysis of Cerebrospinal Fluids (2008-), Paroxysmal Nocturnal Haemoglobinuria screening by flow cytometry (2010-), Minimal Residual Disease (MRD) evaluation in Multiple Myeloma (2017-) and Acute Lymphoblastic Leukemia (2019) and Multiple Myeloma, and Screening of Mature T-cell neoplasm (2023-).

Because therapeutic interventions depend on accurate laboratory assessment it is extremely important to have in place both internal and external quality systems that can measure the quality and reproducibility of the results generated and facilitate appropriate corrective action. The evaluation of the results of the programme during these 25 years. Identified potential sources of variability that might affect the quality of the laboratory results. Selection of appropriate monoclonal antibody clones and fluorochromes showed to be critical particularly for the identification of dim markers. Accordingly, detection of CD22 in chronic lymphocytic leukemia or CD25 in mantle cell lymphomas systematically dependent on the clone-fluorochrome conjugated used. Together with the technical factors, the interpretation of the results might be also affected by other issues. For MRD and samples with low infiltration (eg. cerebrospinal fluids) the number of pathogenic events analyzed was the most significant parameter and, when the aberrant clone was below <0.01% of total cells or less than 50 events, more than one-third of participants failed to detect the infiltration. In addition, experience of the operator was also directly correlated with the performance of the participants, and reproducibility of the results was significantly better in those participants that have completed at least one year of participation in the EQA programme.

In summary, participation in EQA is not only a regulatory obligation but an opportunity to identify technical and analytical artifacts that influence results reproducibility, increasing the efficiency and cost-effectiveness of laboratory test and improving the flow cytometry diagnosis at individual laboratories.

May 15th
Congress

PLENARY SESSION
FLOW CYTOMETRY ADVANCES IN PEDIATRIC SOLID TUMORS

COMMERCIAL SESSION - BECKMAN COULTER
CYTOFLEX MOSAIC: SPECTRAL FLOW CYTOMETRY MEETS MODULARITY

SUPERRCA - INTEGRATING FLOW CYTOMETRY WITH MOLECULAR BIOLOGY FOR THE ULTRA-SENSITIVE DETECTION OF RARE MUTATIONS

COMMERCIAL SESSION - SYSMEX
PRACTICAL DIAGNOSIS OF PNH, WHAT'S NEW IN 2025?

PARALLEL SESSION I
HEMATOLOGY
Measurable Residual Disease

PARALLEL SESSION II
BIOTECHNOLOGY
Flow Cytometry in Human and Animal Research

PARALLEL SESSION I
IMMUNOLOGY
Flow Cytometry in Non-Hematological Disease

PARALLEL SESSION II
BIOTECHNOLOGY
Functional FCM Applications

PLENARY SESSION
SIC WORKING GROUPS & GECLID
Update of activities, user's meeting

FLOW CYTOMETRY ADVANCES IN PEDIATRIC SOLID TUMORS

Chair: Bruno Fernandes, Gabriela Martins

Elaine Sobral
Rio de Janeiro, Brasil

In the world, it is estimated that 400,000 new cases of cancer in children and adolescents each year (Ward et al. 2019). Early diagnosis is fundamental, once it is associated with a more favorable prognosis. (Lan et al., 2019). However, some factors contribute to the time consuming to the diagnosis. First, clinical manifestations can have a substantial intersection with other common childhood diseases, leading to a delay in the cancer suspicion. Next, due to morphological similarities among different pediatric tumors entities - most of them are "blue round cells tumors" at histopathology (HP) examination -, immunohistochemistry (IC) is mandatory to be done, and even molecular tests are necessary in many cases, consuming more days of investigation. Recently, multiparametric flow cytometry (MFC) has been proposed to shorten the time from suspicion to diagnostic, once it is a fast tool, which can evaluate several proteins simultaneously in thousands or millions of cells tumors in a few minutes. Probably due to the large utility of MFC in hematological diagnostics, the first immunophenotypic descriptions of pediatric solid tumors cells were done in bone marrows with leukemia suspicion.

During the last 15 years, EuroFlow has been routinely analyzed mechanically disaggregated tumor cells from masses/solid tissues by MFC aiming to develop, standardize and validate a new strategy to fast diagnostic of pediatric solid tumors. Non hematopoietic tumor cells systematically expressed CD56 in absence of CD45, profile that can separate such cells than immune infiltrated cells on tumor samples. Together with other makers, it allowed the discrimination between tumor and reactive samples, as well as between hematological and non-hematological malignancies with high accuracy/concordance with HP plus IC (Ferreira-Facio et al., 2013).

Next, EuroFlow has developed, standardized, tested and multicentrically validated operation procedures together with an 8-color combination of 12-monoclonal antibodies (tube) for pediatric solid tumors diagnostic orientation – STOT (CD8-CD99/nuMiogenin/EpCAM-CD4/GD2/CD56/CD19-CD3/cyCD3-CD271/CD45). On top of the discrimination between reactive vs. neoplastic samples, hematologic vs non-hematologic tumors, it permitted a final classification according the world health organization (WHO) for the most prevalent extra-cranial non-hematopoietic solid tumors: neuroblastoma, rhabdomyosarcoma, Ewing sarcoma, and Wilms tumors (Ferreira-Facio et al., 2021). In almost all neuroblastoma cases, tumor cells had a high expression of GD2 and CD56 in absence of CD45, Myogenin, CD99, EpCAM and other hematopoietic markers, CD271 was heterogenous, but never so strong as a sarcoma cells tumors. Among sarcoma tumors cases, those with co-expression of CD271+hi/Myogenin+ were rhabdomyosarcoma, while those with CD99+/CD271+hi were Ewing sarcomas. Further, cases classified as sarcomas "non-rabdomyo" were CD271+/CD99-/Myogenin-. EpCAM was negative and GD2 was heterogeneous in these three types of sarcoma. In the few osteosarcoma cases that we were able to recover sufficient cells to analyze, tumor cells were CD271+hi/GD2+hi/CD99+, but in part of the cases the diagnostic is not possible due to lack of viable cells, suggesting that new methods must be evaluated to process osteosarcoma samples. Wilms tumor cells were characterized by EpCAM+/CD271++ cells, which were negative to GD2, Myogenin and CD99. Conversely, germ cells tumors had not a distinctive immunophenotype stained with STOT except for CD56+/CD45-, so for these cases we can report a non hematopoietic tumor by MFC, but not the final WHO category.

In addition, STOT can be applied in any biological sample with pediatric tumor suspicion, not only tumor masses, but also bone marrow, peripheral blood and other biological fluidics, without need of cell disaggregation. For bone marrow and peripheral blood evaluation, we recommend a previous step of bulky lysis and acquisition of 5 million of events to achieve higher sensitivity of tumor detection. On top of it, STOT tube allows the description of the profile of immune cells infiltrating the tumor. Finally, the intensity of expression of GD2 reported wit STOT tube is potentially useful to guide anti-GD2 therapies. Last, an automated analysis and database on Infinicyt software is in development to facilitate STOT analysis.

COMMERCIAL SESSION

MAIN AUDITORIUM

Marta Lopez
Madrid, Spain

CYTOFLEX MOSAIC: SPECTRAL FLOW CYTOMETRY MEETS MODULARITY

Imagine if you could analyze cells with spectral flow cytometry and access single-cell images all at once. No need for separate experiments. No need for additional instrumentation and no need for complicated software and workflows. This possibility will soon become a reality with the new BD FACSDiscover™ A8 Cell Analyzer. This instrument enables high-parameter experiments reducing variability by confirming the cells you're analyzing are the same cells you are viewing through live visualization. You may even discover new insights that were previously undetectable.

During the first part of the talk, Mark Dessing will guide you through the integrated BD SpectralFX™ Technology, enabling high-parameter spectral workflows, expanded panel size and increased flexibility with 78 fluorescent detectors across five lasers, together with BD CellView™ Image Technology, enabling cell image acquisition so that imaging data can be quantified in real-time. Adding single-cell imaging to a flow cytometry workflow helps address questions that couldn't be previously answered by flow cytometry including spatial and physical characteristics of cells such as morphology and protein localization.

This will be followed by the opportunity to see some of the exciting applications Rui Gardner explored while working with a BD FACSDiscover™ A8 prototype.

Sara Bodbin
Uppsala, Sweden

SUPERRCA - INTEGRATING FLOW CYTOMETRY WITH MOLECULAR BIOLOGY FOR THE ULTRA-SENSITIVE DETECTION OF RARE MUTATIONS

Rarity Bioscience is revolutionizing mutation detection with radically improved sensitivity. Our innovative superRCA technology is the result of decades of research and innovation, enabling ultra-sensitive detection of rare nucleic acid sequences in liquid biopsy samples. This talk will guide you through the formation of superRCA molecules, using a combination of padlock probes and rolling circle amplification, through to the readout of superRCA products via flow cytometry, and finally, what this level of sensitivity means for mutation detection.

COMMERCIAL SESSION

BLUE AUDITORIUM

Enrique Colado
Madrid, Spain

PRACTICAL DIAGNOSIS OF PNH, WHAT'S NEW IN 2025?

Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired ultrarare chronic hematologic disease caused by the deficiency of glycosylphosphatidylinositol-anchored proteins (GPI-APs) on the cell surface due to an acquired mutation of the PIG-A gene in hematopoietic stem cells, leading to an uncontrolled terminal complement activation. Over the last decades, the advent of highly sensitive flow cytometry techniques for the detection of PNH clones has led to the more efficient identification of patients with PNH. In this seminar, we will review the contemporary guidelines for PNH diagnosis and follow up and provide recommendations for the reporting and communication with the treating physicians. It is acknowledged that contemporary guidelines for the diagnosis of PNH are driven by the expectations that clinical flow cytometry laboratories will work to ISO15189 standards or National Regulatory requirements that require documented validation of all tests offered. Furthermore, regulatory bodies and other stakeholders will require validation of methods where flow cytometry methods are used for diagnosis, to monitor responses to therapy, or define clinical endpoints. The complex process of assay development, optimization, and validation processes can be followed by the adoption of standardized strategies that need a minimal verification in the busy clinical laboratories, instead of full validation of "in-house" methods.

Regarding reporting, for the practical purpose of clarifying terminology and harmonizing the reports, the Spanish Haematological Flow Cytometry Group (GECFH) has developed a series of recommendations based on existing literature and the activities of clinical laboratories.

When a new diagnosis and follow up of PNH is made or a therapy is selected this can have life changing and potentially lifelong implications for individual patients, therefore, diagnostic testing with the knowledge that an assay has been through a comprehensive validation process and a clinically meaningful report are critically important. Keeping in mind that the ultimate beneficiary must be the patient, we now have the tools to succeed.

PARALLEL SESSION I

MAIN AUDITORIUM

HEMATOLOGY

Minimal Measurable Disease

Chairs: Sérgio Chacim, Ana Espírito Santo, Maria Dos Anjos Teixeira

Łukasz Sędek
Zabrze, Poland

MEASURABLE RESIDUAL DISEASE IN T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

T-cells fulfill their maturation pathway in the thymus resulting in their differentiation into several subtypes. Abnormalities during this process, including mutations in genes for T-cell receptor subunits, different transcription factors or signaling molecules, when accumulated, may result in development of T-cell acute lymphoblastic leukemia (T-ALL). T-ALL accounts for approximately 15% of childhood ALL and 25% of adult ALL. First immunological classification of T-ALL was established back in 1990's and comprised 4 subtypes. In the current WHO classification, a distinct entity, early T-precursor lymphoblastic leukemia/lymphoma (ETP-ALL) has been distinguished from not otherwise specified T-ALL subtypes. The ETP-ALL represents 10–15% of all T-ALL cases and shares the molecular and immunophenotypic features with malignancies developing from myeloid precursor cells. The blast cells in ETP-ALL show usually cyCD3+, CD7+, CD8-, CD1a-, CD5-/dim immunophenotype, with expression of one or more stem cell or myeloid markers. Additionally, immunophenotype of blasts in ETP-ALL can also meet the criteria of mixed phenotype AL which makes it challenging to distinguish the blasts from e.g., myeloid precursor cells or other CD34+/CD7+ precursors.

Measurable residual disease (MRD) is defined as any number of blasts that endure the therapy and are detectable in peripheral blood (PB) or bone marrow (BM) of patients. It is currently well-evidenced that MRD level is a robust marker for the evaluation of treatment quality, with a significant impact on subsequent patient outcome. Information on actual MRD level at defined time points is important in many worldwide AL treatment protocols and is used to make key clinical decisions including risk-stratification of T-ALL patients and possible intensity modification. Detection and quantification of MRD can be done with flow cytometry (FC) or molecular (PCR-based) techniques. However, the use of PCR is not always possible due to the lack of clonal IG/TR rearrangements even in up to 20% of T-ALL patients, particularly ETP-ALL subtype.

Importantly, it is critical to reach the sufficiently high sensitivity. In this context, it is important to emphasize that classical ≥4-color FC-MRD approaches in ALL patients that do not reach sensitivities down to 10-4 are not always reliable, and current treatment regimens require evaluation of MRD with at least 10 4 or higher sensitivity levels. In a fraction of samples this can be technically demanding and to overcome this, several approaches can be proposed: 1) acquisition of high number of cells, 2) increasing the number of parameters (colors) in the FC-MRD assay, and 3) most importantly, designing a robust multicolor FC-MRD panels containing markers that increase the specificity of MRD detection and enable better discrimination between malignant cells and normal cells present in the sample.

Typically, blasts in T-ALL express immaturity markers such as CD34, terminal deoxynucleotidyl transferase, in combination with cytoplasmic CD3, usually without or partial expression of this molecule on the surface membrane, CD10, CD99 and/or CD1a. Other typical T- and/or NK-cell markers, such as CD2, CD4, CD5, CD7, CD8 and CD45 can be expressed on blasts and mature T/NK-cells. Even though T-ALL blasts can differ in expression level of these markers at diagnosis, as compared to mature T/NK-cells, the stability of these markers during treatment is variable, making them insufficient for MRD monitoring. This is why alternative markers of T-ALL blasts are highly desirable in the field and seeking of them became one of the most important goals for many scientific groups. One of such extended multicenter studies was performed within the EuroFlow Consortium which resulted in development of a novel 2-tube, 12-color, 19-marker panel for FC. The EuroFlow approach proved the utility of the panel for high-sensitive MRD detection in virtually all T-ALL patients, and the proposed novel markers and marker combinations improved the discrimination of the blasts from normal cells both in ETP-ALL and non-ETP-ALL patients.

MRD monitoring in T-ALL can be challenging because of the need of intracellular staining. This can bring some troubles in data interpretation related to background increase, and produce both false positive and false negative results. It is also important to choose appropriate clones of monoclonal antibodies for intracellular (e.g., CD3) staining, assuring proper epitope recognition.

Bruno Paiva
Pamplona, Spain

MEASURABLE RESIDUAL DISEASE IN MULTIPLE MYELOMA

Measurable residual disease (MRD) assessment is, from the methodological point of view, ready for prime time in multiple myeloma (MM). Abundant evidence underscores the value of MRD status determined using highly sensitive next-generation flow cytometry and next-generation sequencing tests in evaluating response to treatment and, therefore, prognosis in patients with this disease. MRD response assessment and monitoring might present a range of opportunities for individualized patient management. Moreover, the considerable amounts of high-quality and standardized MRD data generated in clinical trials have led to the acceptance of MRD negativity as an early end point for accelerated regulatory approval of treatments for MM. The data leave no doubt that the efficacy of new regimens in inducing deeper and durable MRD-negative responses is connected with prolonged survival. Yet, several evidential, technical and practical challenges continue to limit the implementation of MRD-guided treatment strategies in routine practice, and the use of MRD as a surrogate end point remains controversial to some. In my presentation, I draw on past and present research to propose opportunities for overcoming some of these challenges, and to accelerate the use of MRD assessment for improved clinical management of patients with MM.

Joana Caetano
Lisbon, Portugal

MEASURABLE RESIDUAL DISEASE IN CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is the most prevalent leukemia in adults in Western countries and is characterized by a heterogeneous clinical course ranging from indolent disease to rapidly progressive forms. Therapeutic advances, particularly the development of targeted agents such as BTK and BCL2 inhibitors, have significantly improved outcomes and shifted the focus from traditional prognostic markers to more dynamic tools, including measurable residual disease (MRD).

In CLL, MRD has emerged as a powerful prognostic biomarker, with undetectable MRD (uMRD) strongly associated with improved progression-free and overall survival. Its utility has been demonstrated across treatment modalities and is gaining traction as a surrogate endpoint in clinical trials, supporting earlier assessment of therapeutic efficacy.

Several techniques are available for MRD detection. Among these, next-generation flow cytometry (NGF), particularly the standardized EuroFlow approach, offers an optimal balance of sensitivity (up to 10^{-5}), specificity, turnaround time, and feasibility for routine monitoring. NGF uses optimized multicolor antibody panels, standardized protocols, and software-assisted analysis to provide reproducible, high-sensitivity detection of residual disease in both blood and bone marrow. It is particularly well-suited for real-time clinical decision making in specialized laboratories.

The role of MRD has expanded in the context of targeted therapies. Venetoclax-based regimens often achieve deep remissions and high uMRD rates, making MRD a practical tool for guiding fixed-duration therapy. BTK inhibitors, while typically inducing prolonged disease control without uMRD, show enhanced MRD clearance when combined with venetoclax. Clinical trials such as MURANO, CLL14, and CAPTIVATE have demonstrated that MRD-guided treatment strategies can safely enable therapy discontinuation and response-adapted approaches. MRD is now an integral part of trial design, increasingly used to guide therapy duration, escalate or de-escalate treatment, and as an exploratory endpoint for regulatory purposes. However, broader implementation in clinical practice requires harmonization of testing methods, clearer guideline integration, and collaboration between laboratories and clinicians.

Looking ahead, the continued standardization of NGF protocols, adoption of AI-based analysis tools, and integration of MRD into real-world practice will be essential for realizing the full potential of MRD-guided care. As the field advances, clinical flow cytometry will remain central to enabling precise, personalized treatment strategies in CLL.

BIOTECHNOLOGY

Flow Cytometry in Human and Animal Research

Chair: Carlos Palmeira , Jose-Carlos Segovia Sanz

Rui Gardner
New York, USA

DEVELOPMENT OF A CUSTOMIZABLE SPECTRAL FLOW CYTOMETRY MOUSE BACKBONE PANEL FOR IMMUNE SURVEILLANCE IN NORMAL AND TUMOR TISSUES

In vivo models are essential for advancing our understanding of cancer biology and immune responses. In this context, multi-parameter flow cytometry enables detailed profiling of immune cells in both tumors and hematological settings. However, designing versatile panels that accommodate the diversity of immune populations across multiple murine tissues remains a complex task. To support this effort, we developed a fixed 14-marker spectral backbone panel that identifies the major immune cell populations and can be expanded to include up to seven additional custom markers [1] — surface or intracellular — tailored to specific experimental questions. The backbone panel delivers consistent and reproducible immune profiling across different spectral flow cytometers and a range of sample types, including hematopoietic, non-hematopoietic tissues, and tumors with complex immune microenvironments. This flexible and robust backbone panel offers a streamlined, cost-effective approach for high-dimensional immune profiling. Its compatibility with validated drop-in fluorochromes supports rapid customization while maintaining standardization, making it an ideal tool for immuno-oncology research and a model for panel development in other research areas.

References:
[1] Longhini et. al (2024) “Development of a customizable mouse backbone spectral flow cytometry panel to delineate immune cell populations in normal and tumor tissues” https://doi.org/10.3389/fimmu.2024.1374943

Òscar Fornas
Barcelona, Spain

SEE WHAT YOU SORT WITH REAL-TIME IMAGING SPECTRAL FLOW CYTOMETRY

Flow cytometry is currently undergoing a major transformation. After the shift to spectral cytometry, the field is now seeing the integration of imaging capabilities. Although imaging is not new to flow cytometry, having been available for years in high-resolution platforms such as the ImageStream, now from Cytek Biosciences, imaging modules are now being incorporated into cell sorters, such as the CellView system in the FACSDiscover S8 from Becton Dickinson. This technology allows not only for the visualization of analyzed events but also for verification of sorted populations. In this presentation, we will showcase several examples where image-based cytometry has provided clear advantages when integrated into a cell sorter. Specifically, we will share our experience with the FACSDiscover S8, which has enabled us to resolve longstanding issues and make significant progress in the study of biologically relevant models. Furthermore, this technology provides valuable morphological insights into the analyzed particles, which is particularly beneficial in studies of microbiome biodiversity or tissues with heterogeneous cellular morphologies.

Jordi Petriz
Barcelona, Spain

SYNERGY FOR PRECISION MEDICINE: THE POWER OF COMBINING LIVE-CELL IMAGING WITH ACOUSTIC FLOW CYTOMETRY

Acoustic flow cytometry imaging represents a significant advancement in cell/particle analysis by employing true brightfield imaging of precisely focused cells through sub 100 nanosecond light pulses that freeze motion with a sensitive, high speed, high resolution monochrome camera. This approach overcomes limitations inherent in other flow imaging systems and traditional camera-free flow cytometry. This combination of acoustic focus, ultrafast light pulses and high-speed camera provides superior image resolution and detail. This results in sharper and more accurate morphological analysis without having to sacrifice the high-speed acquisition of conventional scatter and fluorescence flow parameters collected along with the cell images.

In contrast to camera-free flow cytometry, which uses indirect measurements of light scattering to estimate cell size and complexity, acoustic flow cytometry imaging provides direct visualization of individual cells through brightfield images. This capability enables detailed morphological analysis, encompassing cell size, shape, granularity, and even subcellular features, which is key for accurate cell type identification and the detection of

subtle cellular abnormalities relevant to clinical conditions. Moreover, the ability to capture images allows for the unambiguous confirmation of cell identity from artifacts and the precise discrimination of true single cells from aggregates, a common source of error in traditional flow cytometry. By visually identifying and excluding doublets or higher-order structures, the accuracy and reliability of downstream quantitative analyses are significantly improved. This is really important for applications like Measurable Residual Disease (MRD) and rare cell analysis, where the ability to confidently identify and characterize a small number of target cells within a large background population is critical. Beyond simple intensity measurements, imaging flow cytometry reveals cell details, and cell-cell interactions that are entirely lost in camera-free systems. The wealth of image-based data generated by acoustic flow cytometry imaging is ideally suited for sophisticated analysis using Artificial Intelligence (AI) algorithms. AI can be trained to automatically extract complex morphological features, classify cells into distinct populations, and identify subtle phenotypic variations that might be missed by manual gating in traditional flow cytometry workflows. This AI-powered analysis, integrating both phenotype/function (fluorescence) and structural (brightfield) information at the single-cell level, unlocks the potential for discovering novel diagnostic and prognostic biomarkers and gaining deeper insights into the intricate cellular mechanisms underlying health and disease, establishing acoustic flow cytometry imaging as a powerful and versatile tool for impactful clinical research, particularly in challenging applications like MRD detection and rare cell characterization.

André Mozes
Lisbon, Portugal

THE GOOD, THE BAD AND THE BEAUTIFUL - A SPECTRAL CYTOMETRY JOURNEY

Spectral Flow Cytometry is being installed in every flow cytometry lab, especially in biomedical research labs and biotech companies. During this presentation some of the new features made available by these equipment will be detailed. Nevertheless, as in every groundbreaking technology, some aspects need to be taken into consideration to guarantee the quality of the generated data.

The ability to select and display different autofluorescence profiles is one of the new features that came with this new technology but there are still several aspects that need to be addressed to take the maximum of this great innovation.

PARALLEL SESSION I

MAIN AUDITORIUM

IMMUNOLOGY

Flow Cytometry in Non-Hematological Disease

Chairs: Maria Oliveira, Bruno Fernandes

Sebastiaan Joosten
Amsterdam, Netherlands

SINGLE-CELL PROTEOME PROFILING REVEALS DISTINCT IMMUNOLOGICAL PATTERNS IN THE LUNGS OF PATIENTS WITH ACUTE RESPIRATORY DISTRESS SYNDROME

Rationale:
Acute Respiratory Distress Syndrome (ARDS) is a life-threatening clinical condition caused by diverse etiologies, typically characterized by inflammation and immune dysregulation in the lungs. A comprehensive understanding of the host immune response is essential for identifying distinct ARDS subphenotypes and has significant implications for developing targeted therapeutic interventions. However, existing studies primarily focus on immune responses in the blood. Understanding of local functional heterogeneity has largely relied on transcriptomic data, which gives a comprehensive image but may not translate into phenotypic differences at the protein level. This limitation restricts in-depth insights into immune cell functions within the lung microenvironment. In this study, we describe the alveolar immune composition and function and test differences between etiologies and between survivors and non-survivors.

Methods:
In this observational cohort study, bronchoalveolar lavage (BAL) fluid was collected repeatedly from critically ill patients admitted to the intensive care unit (ICU). Leukocytes were isolated and analyzed using high-dimensional cytometry by time-of-flight (CyTOF). A 50-marker panel was used to assess the maturity, activation states and targeted therapeutic potential of immune cells.

Results:
A total of 128 BAL fluid samples from 91 intubated patients were measured. Of these patients, 75 (82.4%) patients had ARDS, 64 (70.3%) had pneumonia, and 68 (74.7%) patients survived at 28 days post-intubation. Viral ARDS showed a pronounced response, with enriched effector-memory $CD8^+$ T cells expressing granzyme B and perforin and increased CD169^high monocyte-derived macrophages bearing CD80 and HLA-DR. Neutrophil activation was less etiology-specific, remaining uniform across pneumonia subtypes versus non-pneumonia controls. A joint model integrating longitudinal sampling and survival demonstrated that immature neutrophils decreased over time yet were associated with higher mortality, whereas macrophage activation increased with ICU duration.

Conclusion:
Despite the heterogenous etiology of ARDS, patients share similar frequencies of major immune cell populations in the lungs. However, the activation states of these immune cells vary depending on the presence of pneumonia and are associated with mortality.

David Bernardo Ordiz
Valladolid, Spain

UNRAVELLING THE HUMAN INTESTINAL IMMUNOME IN HEALTH AND DISEASE

Mass and spectral approaches have revolutionized the use of flow cytometry, as they provide the chance to perform large (>30-plex) panels. Indeed, and given the great amount of data that these panels generale, in addition to classical or hierarchical gating approaches, unbiased approaches have been developed in order to get the maximum output from these complex panels. Hence, and not surprisingly, computational cytometry has actually taken a central stage for the analysis of complex panels. Indeed, these approaches are currently being performed in several scenarios in order to identify the cellular mechanisms underlaying a given pathology, hence paving the way for the development of novel biomarkers with aid on the clinical practice, hence setting up the bases for novel precision medicine approaches.

Nevertheless, most studies focusing on spectral and computational cytometry usually rely of the study of the peripheral immunome, given the difficulties that studying non-hematological tissues entails. Hence, our group has devoted its research line to the study of the human intestinal immunome in health, as well as in patients with Inflammatory Bowel Disease (IBD), including Crohn´s Disease (CD) and Ulcerative Colitis (UC) using primary samples obtained during the normal course of a colonoscopy (either for disease diagnose or monitoring) or following tissue resection (both from IBD patients as well as from the non-affected tissue from colorectal cancer patients).

Building from that, we have optimized several 40-plex panels using the OMIP-069 (Park et al, Cytometry A, 2020) as a core one. Once applied in the human intestinal lamina propria from non-inflamed controls, our results have shown that, using classical hierarchical analyses, we can identify a total of 52 different lineage immune cell subsets revealing that T-cells (>60%) and NK cells (>20%) were the main populations. Within T-cells, CD4+ and CD8+ were equally represented within both subsets displaying mainly a memory and effector phenotype. NK cells, on the contrary, were largely of the early phenotype. Of note computational cytometry approaches have unveiled a total of 82 different clusters describing as well a unique interindividual immune fingerprint with no inter-regional variations.

Having described the human intestinal immunome in resting conditions, we have also applied spectral cytometry to the inflamed mucosa from IBD patients revealing how, despite the great degree of interindividual variability in health, that nevertheless becomes abrogated when referred to patients with IBD. Hence, UC patients had a total of 35 clusters differentially expressed to controls, while CD patients had a total of 32 clusters. Of note, these clusters were also disease specific since CD and UC patients differed in 15 of them. Last, but not least, and within each disease, the human intestinal immunome also differed based on disease severity revealing a total of 22 clusters differentially expressed in mild Vs. severe UC, and a total of 18 of them between mild and severe CD.

Hence, our results provide novel insight into the study of the human intestinal immunome, not just in health, but also in IBD, hence setting up the bases for future projects that may aim to identify novel immune cell subsets that could set the bases to perform precision medicine in IBD.

José Alexandre Ferreira
Porto, Portugal

TURNING THE GLYCOCALYX INTO CANCER VACCINES: SETTING NEW PARADIGMS FOR CANCER IMMUNOTHERAPY

Cancer cells are enveloped by a dense glycocalyx, a sugar-rich layer composed of glycans attached to proteins and lipids as glycoconjugates. During carcinogenesis, this glycocalyx undergoes extensive remodeling, often resulting in immature glycoforms due to premature truncation in glycosylation pathways. These tumor-associated glycoproteoforms present unique molecular signatures with high potential for biomarker discovery, therapeutic targeting, and clinical decision-making. In this study, we demonstrate how glycoproteomics, in combination with flow cytometry, enables the identification and functional validation of these cancer-specific glycoforms. Focusing on CD44, we identified distinct glycoproteoforms bearing immature O-glycans that correlate with poor prognosis in bladder cancer. Building on this discovery, we engineered a novel class of glycopeptide-based cancer vaccines. Using a chemoenzymatic synthesis and enrichment strategy, we generated libraries of well-defined, tumor-specific CD44 glycopeptides that mirror the heterogeneity of cancer-associated glycans. These were conjugated to immunogenic carrier proteins for enhanced immune activation. Flow cytometry was pivotal in evaluating vaccine performance, enabling high resolution immunophenotyping, tracking both humoral and cellular immune responses, and monitoring changes within the tumor microenvironment. It was also instrumental in immunophenotyping a highly aggressive syngeneic murine cancer model derived from glycoengineered bladder cancer cells expressing the glycoproteoforms of interest. Building on this groundwork, preclinical studies showed that these glycovaccine candidates were well tolerated and elicited robust, long lasting immune responses, including the generation of glycoproteoform-specific antibodies. These antibodies selectively recognized synthetic glycopeptides, glycoengineered cancer cell lines, and patient-derived tumor tissues. In vivo, glycovaccines effectively delayed tumor onset and progression in both prophylactic and therapeutic settings, accompanied by increased immune cell infiltration and activation within the tumor microenvironment. Together, we highlight the transformative potential of tumor-specific glycoproteoforms as both biomarkers and therapeutic targets. By integrating glycoproteomics with flow cytometry, we present a powerful platform for precision cancer vaccines, providing a path from biomarker discovery to functional validation and therapeutic application.

PARALLEL SESSION II

BLUE AUDITORIUM

BIOTECHNOLOGY

Functional FCM Applications

Chair: Enrique O'Connor, Carlos Palmeira

Maria do Céu Monteiro
Gandra, Portugal

FLOW CYTOMETRIC STUDIES ON MODULATION OF PLATELET ACTIVATION RESPONSES WITH CLINICAL APPLICATION

At present, the multifunctional role of platelets is fully recognized. Beyond their involvement in the maintenance of hemostasis, they play a major role during infection and inflammation, immunomodulation, and tumor cell growth or metastasis. Thus, the assessment of platelet activation is crucial in the characterization and diagnosis of platelet-specific disorders, to evaluate the risk for thrombosis or bleeding, monitoring antiplatelet effects and other different conditions of clinical interest in which platelet function could be altered. Flow cytometry is a powerful tool to analyse multiple parameters of platelet activation in a large number of whole blood platelets. In this context, our group has developed and applied a whole panel of platelet activation markers, including earlier and later events of platelet responses, that allows an integrated analysis of platelet activation and platelet-leukocyte interactions. To characterize platelet activation profile, we have designed a panel of biochemical markers, including: intracellular calcium mobilization, pro-coagulant activity, GPIIbIIIa activation, P-selectin (CD62P) expression, ROS generation and platelet-leukocyte interactions. We present here results from different studies where functional flow cytometry has been applied to assess: 1) Genetic factors underlying inter-individual variability in platelet responsiveness 2) Modulation of platelet responses by medicinal herbals 3) Modulation of platelet activation by platelet-rich concentrates used in oral rehabilitation. The results of these studies evidence how multiparametric functional analysis allows the assessment of platelet heterogeneity underlying interindividual variability and thus, contributes to elucidating molecular mechanisms that can help in the establishment of personalized therapies

Alicia Martínez-Romero
Valencia, Spain

IMMUNOLOGICAL AND HAEMOSTASIC STUDIES IN MARINE MAMMALS

Our studies are focused on development and application of flow cytometry assays in marine mammals health. In one hand, we have estudied the platelet function and the platelet microparticles (PMP) formation. Marine mammals may suffer alterations in platelet function and hemostasia due to multiple pathologies, environmental conditions (including stress) or exposure to different contaminants that induce platelet activation. The study of early markers of platelet activation is relevant for the detection, monitoring and therapy of inflammation and hemostasis disorders. Moreover, anthropogenic threats or illnesses could impact blood PMP levels in these animals, We have evaluated the platelet activation, measuring the kinetics of intracellular Ca2+, and the PMP formation, with a real-time phosphatidylserine (PS) exposure assay, in bottlenose dolphins (Tursiops truncatus), beluga whales (Delphinapterus leucas), sea lions (Otaria flavescens) and walruses (Odobenus rosmarus).

In another hand, the study of the immune function in marine mammals is essential to understand their physiology and can help to improve their welfare in the aquariums. Our group is developing new diagnostic and therapeutic tools to promote progress towards preventive medicine in aquariums by facilitating early detection and treatment of diseases. With this objective, we have evaluated the the phagocytic capacity of monocytes and granulocytes in marine mammals whole blood samples from bottlenose dolphins (Tursiops truncatus), beluga whales (Delphinapterus leucas), sea lions (Otaria flavescens) and walruses (Odobenus rosmarus).

PLENARY SESSION

MAIN AUDITORIUM

SIC WORKING GROUPS & GECLID

Update of activities, user's meeting

M. Carmen Martín Alonso
Valladolid, Spain

NEW PROPOSALS IN CELL IMMUNITY QA

Laboratories participating in regular External Quality Assurance for Diagnostic Immunology Labs (GECLID) program demand new external proficiency testing (EPT)Schemes for advanced T and B phenotypes targeting immunodeficiencies. Such EPTs do not currently exist to our knowledge, including B and T populations with specific immunophenotypes of interest that the SIC-SEI Steering Committee is discussing. Neither EPTS targeting cell cycle cytometry and MLPA nor on bronchoalveolar lavage lymphocyte populations. Nowadays the number of cytometry analysis requests and the number of tests to be performed are increasing.

The implementation of those new schemes is conditioned by three factors: (1) a minimum of 5 participants is required to set a robust enough consensus (2) availability of cases allowing us to use their samples with the help of collaborating centres (3) economic viability: either sharing the logistics of regular GECLID shipments, or requesting external funding for a first free pilot run of these schemes, as joint workshops.

A short presentation of a new paper on admissible total error for stem cell determination based on EPT results that has been published last year. The goal is to estimate total error (TE) values for CD34 cell enumeration using state-of-the-art (SOTA) methods with EQA data and to define quality specifications by comparing TE using different cutoffs. The TE measured by EQAS in this study may serve as a quality specification for implementing ISO 15189 standards in clinical laboratories for CD34 cell enumeration.

A summary of 2024 GECLID program performance including data, issues and news will be presented as well. Questions, debate and suggestions on any of the topics in the meeting will be welcome.

May 16th
Congress

PARALLEL SESSION I
IMMUNOLOGY
CAR-T Immunotherapy: An Update

PARALLEL SESSION II
BIOTECHNOLOGY
Flow Cytometry and Extracelular Vesicles: What's new

CLOSING LECTURE
REGULATORY T CELLS FOR THE TREATMENT OF CHRONIC GRAFT VERSUS HOST DISEASE

IMMUNOLOGY

CAR-T Immunotherapy: An Update

Chairs: Alberto Orfão, José Mário Mariz

Julio Delgado
Barcelona, Spain

THE ACADEMIC DEVELOPMENT OF CART-CELLS

The advent of chimeric antigen receptor (CAR) T-cells has improved the outcome of many patients with hematological malignancies and, potentially, solid tumors and autoimmune diseases. However, complex manufacturing and high costs have limited access to these personalized products worldwide. The development of academic products, made by non-for-profit institutions, could enhance the availability of this important technological advances. During the lecture, we will highlight all important key players for such an ambitious goal.

Estefanía Garcia Guerrero
Seville, Spain

ROLE OF FLOW CYTOMETRY IN CAR-T CELL MANUFACTURING AND QUALITY CONTROL

Chimeric antigen receptor (CAR) T cell therapy has transformed the field of immunotherapy, demonstrating remarkable efficacy in patients with relapsed or refractory (R/R) hematological malignancies. Despite its clinical success, the generation of CAR T cells remains a complex and highly regulated process, requiring precise control at each step to ensure safety, efficacy, and reproducibility.

The manufacturing workflow begins with the translation of CAR T cells from preclinical models to clinical-grade products. This transition encompasses three fundamental stages: in vitro and in vivo functional validation, followed by a comprehensive quality assessment under Good Manufacturing Practice (GMP) conditions.

Reliable detection and quantification of CAR T cells are critical throughout the manufacturing process and during patient monitoring. Currently, two primary methodologies are employed: real-time or digital PCR and flow cytometry, each presenting unique advantages and limitations.

Real-time PCR (qPCR) or digital PCR (dPCR) allows for the sensitive and specific quantification of the CAR transgene by amplifying targeted genomic regions. This method offers high reproducibility and is particularly suited for longitudinal monitoring. However, it lacks the ability to characterize the immunophenotype of CAR T cells. Moreover, its sensitivity may be reduced when genomic DNA is extracted from blood samples with a high content of non-T cells.

In contrast, flow cytometry is a single-cell resolution technique that enables the direct detection of CAR molecules via engineered extracellular tags. This method not only permits the quantification of CAR T cells but also facilitates detailed immunophenotypic profiling. Nonetheless, flow cytometry typically demonstrates lower sensitivity than PCR and suffers from a lack of standardized protocols across laboratories.

Accurate phenotypic analysis via flow cytometry requires the optimization and validation of specific antibodies or detection reagents. Multiple commercial reagents are currently available, and their performance may vary depending on the CAR construct and cell product. Indirect detection approaches, such as the use of Protein L, have also been explored. Additionally, some CAR designs incorporate unique markers to facilitate in vitro/in vivo tracking. Given this diversity, validation of each detection method is essential for each individual CAR T cell product.

Overall, flow cytometry plays a pivotal role not only in the characterization and quality control of CAR T cell products but also in their final release for clinical use. Its proper implementation is critical to ensure the safety and therapeutic efficacy of CAR T cell therapies.

Pablo Menéndez
Barcelona, Spain

ADVANCING CAR-BASED IMMUNOTHERAPY FOR CANCER

I will discuss recent achievements with CAR T-cells in lymphoid malignancies and current biological drawbacks in non-B tumors. I will summarize recent preclinical data from our laboratory on how we can advance current CAR T-cell based strategies.

PLENARY SESSION II

BLUE AUDITORIUM

BIOTECHNOLOGY

Flow Cytometry and Extracelular Vesicles: What's new
Chairs: Helena Vasconcelos, André Mozes

Britta A. Bettin
Amsterdam,
Netherlands

STANDARDIZATION OF EXTRACELLULAR VESICLE MEASUREMENTS BY FLOW CYTOMETRY

Extracellular vesicles (EVs) are submicron particles found in body fluids and are promising biomarkers. While flow cytometry is widely used to characterize single EVs, their small size and weak fluorescence and light scattering signals make them difficult to detect. Additionally, flow cytometry data have arbitrary units, which complicates data interpretation, and EV concentrations measured by different FCMs or laboratories are often not comparable. To enable meaningful comparison of EV measurements across assays, FCMs, and laboratories, standardization is essential. This includes (i) using assay controls to confirm that signals originate from EVs, (ii) calibrating the fluorescence and light scattering signals, and (iii) transparent reporting of pre-analytical procedures, experimental and analytical details, and data.

Óscar Fornas
Barcelona, Spain

CHARACTERIZATION AND ISOLATION OF EXTRACELLULAR VESICLES BY FLOW CYTOMETRY

In recent years, flow cytometry has evolved into spectral cytometry, leading to a technological revolution. This is not only due to its extraordinary ability to simultaneously combine dozens of fluorochromes (making it useful in immunological assays) but also to its high sensitivity and resolution. This advancement has represented significant progress in the study of nanoparticles such as extracellular vesicles. Now is possible to identify, characterize, and isolate different subtypes to analyze their content, which is useful in biomarker research, such as in the study of cancer or metastasis, among others, as well as in understanding cellular communication mechanisms. Consequently, flow cytometry has become an essential tool in the study of extracellular vesicles.

Alfonso Blanco
Dublin, Irlanda

R-EV-ELING IN THE NEW TOYS AND COMPLEMENTS

Flow cytometry, originally developed for mammalian cell analysis, has evolved into a versatile tool capable of addressing a wide array of applications, from non-mammalian and plant cells to nanoparticles. With recent advancements in sensitivity and resolution, modern cytometers can now detect particles as small as 40 nm, including bacteria, extracellular vesicles, and synthetic materials like polystyrene beads.

Despite these advances, nanoscale analysis presents distinct challenges that demand careful sample preparation and workflow optimization. This talk will provide practical tips and techniques for the reliable identification, characterization, and sorting of extracellular vesicles—insights that are equally valuable for researchers working with nanomaterials and microorganisms.

Instrumental progress must be supported by parallel improvements in standardization, including calibration materials, fluorescent dyes, and validation protocols. Furthermore, data generated through flow cytometry must be cross-validated with complementary techniques that are evolving alongside the field's expanding requirements.

A couple of examples will be presented to highlight the importance of collaboration between researchers and manufacturers, exemplify how joint efforts can accelerate innovation and address the growing needs of the extracellular vesicle research community.

CLOSING LECTURE

MAIN AUDITORIUM

REGULATORY T CELLS FOR THE TREATMENT OF CHRONIC GRAFT VERSUS HOST DISEASE

Chairs: Julia Almeida, Jose-Carlos Segovia Sanz

João Forjaz de Lacerda
Lisboa, Portugal

Hematopoietic stem cell transplantation (HSCT) is an important curative treatment for many patients with severe diseases of the hematopoietic system. Severe chronic graft versus host disease (cGVHD) remains a major cause of morbidity and mortality after HSCT. cGVHD has a complex pathobiology, where impaired T-cell homeostasis plays an important role. This directed us to prospectively study in detail Treg, Tcon (non-Treg CD4 cells) and CD8 homeostasis in patients submitted to unrelated donor HSCT and found that patients developing cGVHD have decreased true naïve and stem cell memory Tcon and Treg, and increased true naïve, SCM and EMRA CD8+ cells early post-transplant. These results suggest that these imbalances in the T-cell compartment and that the loss of immunologic tolerance associated with poor Treg recovery are likely associated with the pathobiology of cGVHD. The observation that Treg were decreased in patients with cGVHD led us to design a Phase I/II clinical trial infusing fresh purified Treg from the original HSCT donor into patients with moderate and severe cGVHD. These patients traditionally need to be treated with a combination of immunosuppressive drugs that increase their risk for severe infections. This study was performed under the auspices of the Horizon 2020 EU-funded TREGeneration Project that we coordinated in Lisbon, which brought together several groups in Europe investigating Treg therapies for cGVHD. Despite the promising results of this trial, in trying to move the field forward, we have been able to select and expand in vitro Treg after cultures of highly purified Treg with dendritic cells from an HLA-matched sibling to generate a more potent and specific Treg product. We are currently studying the specificity, potency and mechanisms of suppression of these cells, with the aim of bringing this therapy into a clinical platform.

05. Abstracts

Short Communication
ePosters
Publications

Abstracts

Short Communication

Title DEVELOPMENT AND ACCREDITATION OF SPECTRAL CYTOMETRY PANELS: ADVANCES IN THE DIAGNOSIS AND MONITORING OF LEUKOCYTE MALIGNANCY **Code** 23

Authors Names Alfredo Minguela (1); Yazine Kharraz (2); José Antonio Campillo (1); Ruth López-Hernández (1); Inmaculada Ruiz-Lorente (1); María José Alegría Marcos (1); Alicia Hita-Ruiz (1); Marina Fernández (1); Rosana González-López (1); Carlos Carrascoso (3)

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Topic Hematology

Abstract

Introduction Flow cytometry immunophenotype studies are a complementary yet fundamental tool in the diagnosis and monitoring of chronic and acute leukocyte malignancies. The objective of the flow cytometry study, whether analog, digital, or spectral, is to define a leukemia-associated immunophenotype (LAIP) useful for diagnosis and measurable residual disease (MRD) studies. This requires defining a sufficiently broad and pathology-specific antigenic panel to identify cellular lineage, maturation stage, and -most critically- abnormal marker expression.

Methods Panels were designed for use with the Cytek-NL cytometer equipped with 405 nm, 488 nm, and 640 nm lasers. Fluorochrome selection prioritized antigen expression levels, compatibility indices, and spread using the SRI matrix. Biological fluid and tissue samples from healthy donors and patients with diverse pathologies were analyzed. Monoclonal antibodies were sourced from multiple manufacturers. Data acquisition was done with SpectroFlo software (Cytek), and analysis with FACS-Diva 9.0 (BD Biosciences). To streamline workflow and minimize errors, antibody cocktails were stored as frozen aliquots at -20°C.

Results Validated panels: 1) MYELOID PATHOLOGY PANEL, 25 fluorochromes targeting 25 markers (CD3, CD4, CD7, CD10, CD11b, CD13, CD14, CD16, CD19, CD33, CD34, CD36, CD38, CD43, CD45, CD56, CD64, CD66abce, CD71, CD105, CD117, CD123, CD157, CD300e, HLA-DR); 2) CHRONIC LYMPHOPROLIFERATIVE SYNDROMES PANEL, 23 fluorochromes targeting 23 markers (CD3, CD4, CD5, CD7, CD8, CD10, CD11c, CD19, CD20, CD23, CD25, CD38, CD43, CD45, CD56, CD103, CD305, CD200, FMC7, Kappa, Lambda, TCRγδ, TCR-cβ1); 3) B ACUTE LYMPHOID LEUKEMIA PANEL, 22 fluorochromes targeting 22 markers (CD3, CD4, CD10, CD13, CD19, CD20, CD22, CD24, CD33, CD34, CD38, CD45, CD45RA, CD56, CD58, CD66abce, CD73, CD81, CD117, CD123, CD304, HLA-DR); and 4) T ACUTE LYMPHOID LEUKEMIA PANEL, 22 fluorochromes targeting 22 markers (CD1a, CD3, CD3cyto, CD4, CD5, CD7, CD8, CD10, CD16, CD19, CD33, CD34, CD38, CD48, CD45, CD56, CD66abce, CD99, CD117, TCRαβ, TCRγδ, HLA-DR). The results of the panels will be described in more detail in the presentation of this abstract.

Conclusion Spectral cytometry panels were validated against 12 color panels with 12 to 17 markers in digital FACS-Lyric cytometers using diverse biological samples. Frozen aliquots remained stable for over one month at -20°C. These panels enable identification of diverse pathological phenotypes in chronic/acute myeloid and lymphoid malignancies, proving high-resolution and sensitivity for both diagnostic and MRD studies.

Conflict of Interest No.

Title HARNESSING THE POWER OF LEMON BY-PRODUCTS: FLOW CYTOMETRY CONFIRMATION OF THEIR ANTIBACTERIAL ACTIVITY **Code** 34

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Topic Microbiology

Abstract

Introduction Citrus is one of the most widely cultivated fruit crops and one of the main consumed products in the Mediterranean area. The annual production of different citrus fruits was approximately 143 thousand tonnes, whereas lemons and limes represented around 20 thousand tonnes in 2019. Industrial processes exploit only 45% of the total fruit weight, which generates a significant amount of waste, including peel (flavedo: 27%), pulp (albedo and endocarp: 26%), and seeds (2%). Lemon by-products are composed of significant amounts of bioactive compounds, which give them bioactivities related to food preservation, such as antibacterial capacity. Furthermore, lemon by-products, which are usually discarded as waste in the environment, can generate new ingredients, such as essential oil (LEO) and lemon phenolic compounds-rich extract (LPC), being an opportunity for the food industry to promote the zero-waste concept.

Methods To understand the preservative potential of these ingredients, the antibacterial capacity (minimal inhibitory concentration, MIC) was first assessed. In addition, flow cytometry was performed using the BDTM Cell Viability Kit (Microbial Viability Measurements on the BD AccuriTM C6 Flow Cytometer). This kit provides an easy-to-use dye combination that distinguishes live and dead cell populations. The thiazole orange (TO) technique confirms the enumeration of live and dead bacteria, allowing discrimination of cells from background electronic noise or debris. Propidium iodide (PI) is impermeable to healthy cells with intact membranes but permeates cells with compromised membranes, such as dead cells. These dyes provide a rapid, simple method to differentiate between live and dead bacteria when used together. Simultaneous TO and PI staining allows for the distinction among live (TO+PI–), dead (TO+PI+), and injured (TO+PIint) cell populations.

Results Among the ingredients, LEO is recognised for its inhibition potential against bacteria, with an MIC of 31.25 uL/mL for Escherichia coli and 62.5 uL/mL for Pseudomonas aeruginosa. The flow cytometry for LEO was accessed, and for E. coli, dead (42.3%), live (15.1%), and damaged cells (37.7%) were observed; and P. aeruginosa showed dead (71.3%), live (2.4%), and damaged cells (20.0%). Furthermore, the LPC also presents a good antibacterial capacity, with a MIC of 62.5 uL/mL for Staphylococcus aureus and 31.25 uL/mL for P. aeruginosa. The flow cytometry for this functional ingredient for S. aureus showed dead (62.2 %), live (1.7%) and injured (34.9%), and P. aeruginosa showed dead (76.6%), live (37.1%), and damaged cells (12.9%).

Conclusion Based on these results, it can be concluded that lemon co-products contain interesting bioactive compounds with antibacterial properties and serve as a suitable matrix for extracting functional ingredients. This significantly supports their valorisation in food applications for extending the shelf-life of food products and presents an innovative idea for a changing world.

Conflict of Interest No.

Title IMMUNOLOGICAL BIOMARKERS OF CVID SURVIVAL **Code** 5

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Topic Hematology

Abstract

Introduction Common variable immunodeficiency (CVID) comprise a heterogeneous group of patients with hypogammaglobulinemia suffering from infectious and non-infectious complications that might lead to severe organ-damage and shortened survival. Appropriate clinical management of CVID has been hampered by the lack of robust biomarkers to predict the development of clinical complications and patient outcome.

Methods Blood samples were collected from 166 CVID patients at 16 different centers in Europe. CVID patients were classified according to memory B-cell/plasma cell defects (Euroflow CVID0-6), naïve CD4+ T-cell counts (late onset combined immunodeficiency -LOCID-), pathogenic/risk alleles, and serum antibody levels at baseline or study enrolment. Median follow-up was 5 years.

Results CVID patients presenting with non-infectious complications showed a shortened 5-year overall survival (OS: 87% vs. 100%, p=0.03, respectively) as compared to cases with infections-only. When CVID patients with non-infectious complications were analyzed separately, 5-year OS rates were significantly lower (p≤0.004) in those with LOCID (HR: 8), CVID5-6 (HR: 18), and baseline serum IgG0.05); 2) intermediate-risk - CVID0-4 patients with non-infectious complications who simultaneously showed LOCID and ba

Conclusion Detailed evaluation of B-cells and naive TCD4 cells might contribute to identify patients at increased risk of shortened survival who could benefit from more intensive therapeutic interventions (e.g., allogeneic hematopoietic stem cell transplantation).

Conflict of Interest No.

Title REPRODUCIBILITY OF NON-IVD-R CERTIFIED SPECTRAL FLOW CYTOMETERS IN MULTICENTER SETTINGS: IMPACT FOR APPLICABILITY IN COLLABORATIVE CLINICAL RESEARCH **Code** 55

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Topic Others

Abstract

Introduction High-dimensional spectral flow cytometry has revolutionized cellular immunophenotyping. The increasing number of publications showcasing optimized, high-parameter panels highlights the technology's power. However, current IVD-R certified spectral flow cytometers (SFC) are limited to three lasers, restricting panel complexity and the depth of information obtainable. Consequently, multiple studies employ non-IVD-R instruments to achieve the necessary analytical depth. However, the reliance on these instruments, especially in multicenter studies, requires rigorous evaluation of inter-instrument comparability to ensure data reproducibility and accurate interpretation, crucial for reliable interpretation and translation of research findings into clinical applications. Therefore, this study aimed to evaluate the reproducibility of non-IVD-R certified SFC in multicenter/multiplatform settings.

Methods Four 5-laser SFC (1 Aurora CS cell sorter, 3 Aurora cytometers, Cytek) were assessed in 3 centers (Salamanca, Milan, Utrecht). Instrument detector performance was determined by evaluating the raw spectra of fluorescent beads and 21-38 different fluorochromes. For cell sorter (CS) vs. cytometer (CYT) comparison, a 20-marker panel designed for broad immune cell profiling, was used. Processed samples were analysed on both instruments to evaluate the influence of sample flow rate input on marker resolution (staining index) and overall staining reproducibility, enabling a direct instrument comparison. Multicenter CYT comparability was evaluated using peripheral blood samples from 9 healthy adults, locally collected and processed with a 43-marker T cell-oriented panel and centrally distributed antibodies to minimize variability due to different reagent lots.

Results Raw spectra of fluorescent beads and fluorochromes showed a strong correlation between the CS and CYT (median fluorescence intensity (MFI) differences 0.98 for 18/20 fluorochromes). Marker resolution differences exceeding 30% were observed for 6/20 markers. Both CS and CYT platforms showed stable raw data MFI at lower flow rates. However, sample input rates ≥30µL/min led to a minor (4.6±4.3%) decrease in MFI and increase in the robust coefficient of variation (%rCV) for the detectors from all laser lines, except the ultraviolet, on the CS, while the CYT platform maintained stable MFI and low %rCV (systematically ≤6%). Higher sample input flow rates decreased marker resolution on both instruments, with a greater reduction observed on the CS (31.8% vs. 12.9% at ~60µL/min). Despite these differences, multivariate analysis of 22 populations revealed high sample overlap, indicating negligible instrument or flow rate effects on overall population analysis.
Multicenter CYT evaluation revealed excellent raw spectra comparability (R>0.99, bias30% MFI variation (positive reference population), multivariate analyses (FlowSOM, PCA) confirmed high inter-center comparability, with 23/24 populations classifying within 2 standard deviations of the Salamanca reference.

Conclusion Despite minor instrument and flow rate-dependent variations, non-IVD-R spectral flow cytometers demonstrated robust comparability. These results validate their reliable use in collaborative clinical studies for high-dimensional immunophenotyping, given appropriate standardization and quality control. Funding: USAL4EXCELLENCE MSCA-COFUND fellowship (European Union's Horizon 2020 research and innovation programme; grant agreement 101034371). Andrés Laguna fellowship (Junta de Castilla y León, co-financed by the Fondo Social Europeo).

Conflict of Interest Yes. J.P. is also an employee of BD Biosciences.

Title DETECTION OF CIRCULATING TUMOR PLASMA CELLS AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION IN MULTIPLE MYELOMA PATIENTS: PREDICTIVE VALUE FOR ANALYTICAL PARAMETERS ALTERATIONS AND DISEASE PROGRESSION **Code** 60

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Topic Hematology

Abstract

Introduction Measurable residual disease (MRD) assessment in multiple myeloma (MM) is crucial for monitoring treatment response and early detection of relapses. In clinical practice, bone marrow (BM) aspirate, analysed by flow cytometry (FC), is widely used as the standard method for this evaluation. However, it is an invasive procedure that may present challenges in patients with bone fragility, a common feature of MM, often exacerbated by intensive treatments like autologous stem cell transplantation (ASCT). Malignant plasma cells can migrate from bone marrow to peripheral blood, allowing their detection. which can constitute a promising, non-invasive, and complementary approach to access the MRD status. This study aims to evaluate the applicability of detecting circulating tumor plasma cells (CTCs) by FC, in peripheral blood from MM patients, comparing the results with those from BM and positron emission tomography and computed tomography (PET-CT), while also assessing its relationship with some analytical markers.

Methods A total of seventy-seven patients diagnosed with MM and submitted to ASCT, with a mean age of 65.3 years (37 females and 40 males), underwent continuous follow-up with a median follow-up of 6 years. Forty-six patients received maintenance therapy, primarily with lenalidomide; among them, 9 also received thalidomide. Peripheral blood samples (6–9 mL) were collected in EDTA tubes and processed by FC within 24h after collection. CTCs assessment was performed by FC, employing a bulk lysis protocol designed to maximize nucleated cell recovery with the purpose of targeting a total of 10 million events per sample. The analysis was conducted using the second tube of the standardized EuroFlow MM panel, applied on an 8-color BD FACSCanto II cytometer. The monoclonal antibody panel included: CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCP Cy5.5, CD19-PE Cy7, cytoplasmic light chain kappa -APC, and lambda APC-C750. Data acquisition was performed using FACSDiva software, in accordance with EuroFlow standard operating procedures, including fluorescence compensation. The results of CTCs detection were correlated with routine analytical markers, including albumin, urea, creatinine, haemoglobin, calcium, lactate dehydrogenase (LDH), K/L free-light chain ratio, and beta-2-microglobulin (B2). An ANOVA test was used to assess statistical differences in the mean values of these markers between patients with and without detected CTCs. Additionally, a linear regression analysis was performed to evaluate the predictive value of CTCs detection for subsequent changes in the studied analytical parameters, as well as for potential later relapses.

Results CTCs were detected in 11 of the 77 studied patients, with a median of 2 determinations per patient during the follow-up (range from 1 to 6). A median of 7.8 million events was analysed per sample, with an IQR of 7 to 10 million. The percentage of CTCs detected in peripheral blood among these patients ranged from 0.00024% to 10%. Importantly, all patients with detectable CTCs experienced clinical relapse. When comparing the results obtained in peripheral blood and BM, we observed that 8 patients with negative CTCs, the MRD detection in BM was positive (with a median of 6 million analysed events). Similarly, in 9 cases, PET-CT scans showed positive results, while CTCs analysis performed at the same time yielded negative results (median of 7 million analysed events). In all these discordant cases, less than 10 million cells were analysed in CTCs evaluation, emphasizing the impact of sample quantity (and consequently the number of analysed events) on detection reliability. Conversely, concordant results between CTCs and BM were observed in 8 cases (median of 7.9 million events analysed per peripheral blood sample). Furthermore, in a total of 9 cases with positive CTCs detection, PET-CT results were either negative (n = 6) or revealed small lesions of uncertain clinical significance (n = 3), with a median of 8.5 million cells analysed per peripheral blood sample. Throughout the study, no CTCs were detected in 66 patients. Additionally, significant differences in K/L ratio (p=0.001), haemoglobin (p=0.025), and LDH (p<0.001) levels were observed between patients with and without CTCs. This methodology was also able to identify malignant plasma cells before significant changes occurred in routine analytical markers. Linear regression models showed that creatinine levels exhibited a continuous increase over time (p=0.004), following the initial detection of CTCs.

Conclusion CTCs detection by FC seemed to be an effective and non-invasive technique for detecting CTCs in MM, with a good correlation with BM analysis, particularly when, at least, 10 million leukocytes were analysed. Moreover, CTCs detection consistently preceded clinical relapse, suggesting its potential as an early marker of disease recurrence.

Conflict of Interest No.

Title UNLOCKING MURINE IMMUNE COMPLEXITY: A STANDARDIZED SPECTRAL FLOW CYTOMETRY TOOLBOX FOR HIGH-DIMENSIONAL ANALYSIS **Code** 54

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Topic Animal Sciences

Abstract

Introduction Murine models are a valuable tool for monitoring in vivo immune responses in pre-clinical studies, in multiple settings such as vaccination, infection, autoimmunity, and tumor immunology. However, designing robust spectral flow cytometry (SFC) panels for comprehensive murine immune profiling is complex, considering factors such as tissue-specific cell composition, in vivo stimulation needs, fluorescent reporter compatibility, sample volume limitations, and cost. Therefore, this study aimed to develop a standardized SFC toolbox to enable versatile immune profiling across diverse experimental settings.

Methods For tool development, a three-step approach was employed. An initial selection of a minimum core of non-redundant markers required for cell identification was performed, followed by design of a fixed backbone, and different expansions adjusted to distinct research questions, allowing for comprehensive profiling of innate and adaptive immune cells across various tissues, including blood, spleen, lymph nodes and lung. Lastly, the assay was optimized for sample multiplexing, in vitro stimulation, and transcription factor analysis. Furthermore, the performance of the multiplexed combination was evaluated in a longitudinal study, employing limited sample volume (≤50µL), to determine the immune responses to lipid nanoparticles (LNPs), commonly used in mRNA vaccination models.

Results Overall, a combination of CD25, CD127, and CD304 accurately identified regulatory T cells (R^2>0.9) in blood and spleen. However, no reliable surface membrane markers were found as surrogates for Th1, Th2, Th17, and Th22 T cell identification.
A backbone consisting of CD3, CD19, CD335 and a viability marker, served as the foundation for panel expansion for lymphoid cell studies (up to 29 markers), and exclusion cocktail for myeloid cell analyses (up to 18 markers). An overall combined 38-marker panel allowed for the identification of up to 115 immune cell subsets. This panel included markers for maturation and activation assessment, and was compatible with fluorescent protein reporters (e.g., GFP, EGFP, mCherry, ZsGreen). Additionally, it allowed for the inclusion of additional characterization markers (e.g. M1/M2 markers, such as CD163 and CD80), and evaluation of transcription factors (e.g. T-bet and GATA3). In vitro stimulation in presence of TAPI and brefeldin A allowed for functional T helper subset identification, based on cytokine production profiles, alongside maturation stage characterization. Furthermore, CD45-based barcoding allowed for sample multiplexing, with negligible impact on resolution. Longitudinal immune monitoring (38-marker combination, multiplexed samples, ≤50µL of blood), demonstrated that LNPs elicited a strong immunogenic response with specific cellular kinetics. This response featured early (day +1) innate immune responses, with increased circulating mature and immature neutrophils, intermediate monocytes and conventional type 2 dendritic cells. Subsequently, on day +21 and post booster (day +36), an increase in circulating B1b B cells was observed, suggesting the potential involvement of T cell-independent antibody responses.

Conclusion In this study we developed a standardized SFC toolbox that enables robust, high-dimensional immune profiling in murine models, offering broad applicability for preclinical research by also decreasing the design and optimization burden for researchers. Funding: Grant PID2022-136462NB-I00 funded by "Ministerio de Ciencia e Innovación" and the European Union. CT is a recipient of a USAL4EXCELLENCE MSCA-COFUND fellowship (European Union's Horizon 2020 research and innovation programme; grant agreement No. 101034371) and of the Andrés Laguna fellowship (Junta de Castilla y León, co-financed by the Fondo Social Europeo Plus, FSE+). JS-M is recipient of the predoctoral fellowship program of Junta de Castilla y León, co-funded by "Fondo Social Europeo" (Orden EDU875/2021).

Conflict of Interest Yes. J.P. is also an employee of BD Biosciences.

Title THE IMMUNOSUPPRESSIVE LANDSCAPE OF LUMINAL B-LIKE BREAST CANCER TUMORS **Code** 20

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Topic Immunology

Abstract

Introduction Breast cancer is one of the most prevalent malignancies worldwide and is characterized by significant biological and clinical heterogeneity. Among the molecular subtypes, luminal A and luminal B tumors are the most common, distinguished by the expression of hormonal receptors. Luminal A is typically associated with better prognosis, while luminal B is characterized by higher proliferative potential. The tumor microenvironment (TME) plays a crucial role in disease progression by regulating the immune response through the infiltration of various immune cell populations. T cells play a key role in tumor immunosurveillance, with their distinct functional subpopulations, exerting distinct functions in the anti-tumor response. Given the potential differences in immune cell infiltration between luminal A and luminal B subtypes, this study aims to compare the frequency and distribution of T cells subpopulations within the tumor microenvironment of these breast cancer subtypes.

Methods This study included 40 women diagnosed with breast carcinoma (mean age: 59 ± 7 years), classified into Luminal A (n = 23) and Luminal B (n = 17) subtypes. Tumor samples were obtained through ultrasound-guided breast biopsy and analyzed using flow cytometry to characterize immune cell subpopulations. The T cell panel used include TCR γδ (FITC), CD4 (PE), CD196 (PerCP-Cy5.5), CD127 (PE-Cy7), CD25 (APC), CD27 (APC R700), CD8 (APC-H7), CD185 (PB), HLA-DR (PO), CD3 (BV605), CD195 (BV711) and CD45RA (BV786). Data acquisition was performed on FACSLyric flow cytometer. Comparisons between immune cell subpopulations in the two subtypes were performed using parametric (independent t-test) and non-parametric (Mann-Whitney) tests.

Results The analysis of clinicopathological characteristics revealed significant differences between Luminal A and Luminal B tumors. Luminal A tumors were predominantly grade 1, while Luminal B tumors were more frequent in grade 2. T cell subpopulations showed a significant increase in CD4+ and CD4+CD8+ cells in Luminal B tumors, along with more CD8+ Treg and Th17 cells. In contrast, Th1 cells were more frequent in Luminal A tumors. Principal component analysis (PCA) revealed that most Luminal B tumors clustered together, indicating shared immunological features, while Luminal A tumors showed more immune heterogeneity. Luminal A tumors with higher Ki-67 levels also clustered together, potentially correlating with a more aggressive behavior.

Conclusion These findings, despite being achieved in the two breast cancer subtypes with a better prognosis, highlight that luminal B-like breast cancer tumors seams to possess a more immunosuppressive TME than luminal A. This altered immune profile may impair effective anti-tumor immune responses, offering insight into the immunological differences between these subtypes. These results suggest potential new therapeutic targets and biomarkers for precision medicine in breast cancer, focusing on modulating the immune microenvironment to improve patient outcomes.

Conflict of Interest No.

Title NEUTROPHIL APC-LIKE CELLS: A DISTINCT POPULATION OF LDG WITH UNIQUE FEATURES AND POTENTIAL USE AS A BIOMARKER OF CARDIOVASCULAR RISK **Code** 45

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Topic Immunology

Abstract

Introduction Low-density granulocytes (LDGs) have emerged as critical contributors to the pathogenesis of systemic lupus erythematosus (SLE) and its associated cardiovascular disease (CVD). The heightened activation of LDGs and their enhanced capacity for NETosis contribute to immune dysregulation, vascular inflammation, and the development of atherosclerotic plaque formation, which significantly elevate the risk of cardiovascular events in SLE patients. In this study, we identified a distinct subset of low-density neutrophils exhibiting antigen-presenting cell-like (nAPC-like) features, which are associated with vascular inflammation.

Methods Low-density granulocytes (LDG), monocyte subsets (classical, intermediate and non-classical) and Th1/Th17/Treg lymphocytes were quantified by flow cytometry in freshly isolated peripheral blood mononuclear cells from SLE patients (N=143), healthy controls (N=33) and non-autoimmune atherosclerotic individuals (N=22). High-dimensional clustering and visualization of myeloid cell populations from patients and controls were performed using the FlowSOM algorithm. Circulating levels of these cell immune subsets were analyzed in relation to subclinical carotid atheromatosis, overt CVD, and SLE-specific clinical parameters, including disease activity and ongoing therapies.

Results Conventional two-dimensional flow cytometry revealed a minor population of myeloid cells commonly present in PBMC from SLE patients and controls, which were excluded from standard LDG and monocyte gating strategies due to their simultaneous expression of CD14 and CD15. High-dimensional cytometric analysis confirmed the presence of a distinct $CD14^{+}CD15^{+}CD16^{+}$ population, separate from the previously reported CD16- and CD16+ LDG subsets as well as the three canonical monocyte subsets. Further phenotypic characterization in 15 SLE patients identified these cells as neutrophils ($CD66b^{+}CD15^{+}$) with increased cell size relative to conventional LDGs. These cells expressed CD14 and HLA-DR and included CD10pos/neg and CD16dim/high phenotypes, consistent with features of nAPC-like neutrophils. Although present in both controls and patients, notable differences were observed. In controls, nAPC-like cells positively correlated with carotid intima-media thickness (cIMT) ($p=0.004$) and C-reactive protein (CRP) levels ($p=0.042$) and were elevated in those with traditional cardiovascular risk factors (tCVR) or subclinical atheromatosis compared to their CV-free counterparts. Similarly, these cells were increased and correlated with cIMT ($p=0.013$) in non-autoimmune atherosclerosis group. In SLE patients, nAPC-like cells correlated with CRP ($p<0.001$), SLE Disease Activity Index (SLEDAI) ($p<0.001$), and anti-dsDNA titers ($p<0.001$) and were significantly increased in patients with tCVR or clinical/subclinical CVD. Notably, a minor CD16dim nAPC-like subset, characterized by a CD10negCD66bhigh phenotype and lower CD15 expression than CD16high counterparts, suggested an immature neutrophil status. In patients, this subset correlated with serum of IL-6 and BLyS levels. Finally, total nAPC-like cells showed positive correlations with Th1 ($p=0.006$) and Th17 ($p=0.004$) cell subsets—both increased in SLE relative to controls—while exhibiting a negative association with regulatory T cells ($p=0.010$).

Conclusion Our findings reveal the presence of increased levels of a distinct low-density neutrophil CD14+CD16dim/highHLA-DR+ population with both mature and immature phenotypes in individuals with traditional cardiovascular risk and subclinical atherosclerosis, including those with SLE and healthy donors. The association of these nAPC-like cells with endothelial damage and immune dysregulation suggests their potential utility as early biomarkers of cardiovascular risk and as targets for preventive therapeutic strategies.

Conflict of Interest No.

Title COMPREHENSIVE ATLAS OF MONOCYTIC HETEROGENEITY AND DIFFERENTIATION ACROSS HUMAN TISSUES: A FRAMEWORK FOR CLINICAL TRANSLATION **Code** 56

Authors Names Kyra van der Pan (1); Enrique De La Rosa (2); Indu Khatri (1,3); Sara Kassem (1); Anniek de Jager (1); Brigitta Naber (1); Inge de Laat (1); Alesha Louis (1); Marjolijn Hameetman (4); YK Onno Teng (5); Meta Roestenberg (6); Beatrice MF Winkel (6); Clarize M de Korne (6); Stijn Crobach (7); Wouter BL van den Bossche (8); Daniela Damasceno (9); Alberto Orfao (9); Javier De Las Rivas (2); José Manuel Sánchez-Santos (10); Jacques JM van Dongen (1,9); Paula Díez 1,11); Cristina Teodosio (1,9)

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Topic Immunology

Abstract

Introduction Monocytes (Mo) and macrophages (MAC) play pivotal roles in tissue homeostasis, immune responses, and cancer. Though knowledge of the monocytic system is expanding, the dynamic nature of Mo/MAC maturation, marked by plasticity and functional diversity, and lack of consensus on subset definition, hinder research comparability and clinical application. Therefore, this study aimed to comprehensively elucidate monocyte maturation and heterogeneity across tissues, establishing a framework for clinical translation.

Methods We analysed Mo/MAC maturation and heterogeneity across bone marrow (BM), peripheral blood (PB), skin, peritoneal dialysate (PD), and colon, employing a combination of flow and mass cytometry (CyTOF), mass spectrometry (MS)-based proteomics, morphology, functional assays, and bioinformatics. Initially, >100 proteins were screened to identify key markers of monocyte heterogeneity. A 34-marker CyTOF panel was designed, enabling the evaluation of >15 Mo/MAC populations (n=39). Based on the CyTOF data, 5 BM (n=5), 8 PB (n=5), and tissue MAC (skin: n=4; PD: n=3; colon: n=5) populations were purified for TMT-based MS-based proteomics. Data integration and trajectory inference analyses were performed using bioinformatic approaches to delineate monocyte maturation and inter-tissue relationships.

Results Based on population relationships defined using single cell trajectory analysis, 11 distinct BM protein expression patterns (PEPs) were identified. These PEPs captured monoblast-to-promonocyte transition (e.g., autophagy, mTOR, Wnt signalling downregulation), sequential functional acquisition (e.g., chemokine, C-type lectin receptor and TNF cascades), and variable protein modulation, likely driven by functional regulation, transcriptional control, and signaling pathway activation. CD62L, FcεRI, CD36, and Slan defined eight distinct classical (cMo) and non-classical (ncMo) Mo subsets in PB with unique functional/morphological profiles. cMo showed branched trajectory and significant functional/proteomic overlap, associated with distinct functional specializations: CD62L+FcεRI- (inflammatory; TNF/IL1β production, TLR4 signalling), CD62L-FcεRI- (antiviral; cytosolic DNA sensing, RIG-I-like receptor signalling), and FcεRI+ (mixed pro/anti-inflammatory; IL6/IL13 production), suggesting they may represent distinct activation states and/or specialized functions, rather than sequential maturation stages. Acting as a functional bridge between cMo and ncMo, intermediate Mo exhibited enhanced endocytosis, antigen presentation, immune regulation, and metabolism. ncMo subsets defined by CD36/Slan, exhibited a linear trajectory, associated with decreasing phagocytic ability and progressive functional specialization: immune surveillance (CD36+Slan-), stress response (CD36-Slan-), and cellular maintenance and antiviral response (CD36-Slan+). CD36+Slan+ ncMo deviated from the linear ncMo trajectory, showing limited bacterial response but strong antiviral/stress signatures, suggesting they could represent a specialized state. While sharing a core functional identity, MAC displayed tissue-specific adaptations: dermal (M2-like anti-inflammatory profile, high metabolism, antigen presentation), colon and PD (mixed pro-inflammatory/regulatory features).

Conclusion By integrating multi-omics approaches, we delineated monocytic maturation across tissues, shedding light on the complex maturational relationships and functional heterogeneity of these cells, and providing new avenues for clinical applications. Funding: ERC Advanced Grant (ERC-2015-AdG 695655, TiMaScan; European Research Council under the European Union's Horizon 2020 Research and Innovation Programme); USAL4EXCELLENCE MSCA-COFUND fellowship (European Union's Horizon 2020 research and innovation programme, MSCA grant agreement 101034371); Andrés Laguna fellowship (Junta de Castilla y León, co-financed by the Fondo Social Europeo); Ramón y Cajal fellowship (RYC2022-035568-I).

Conflict of Interest No.

Title FUNCTIONAL CHARACTERIZATION OF CAR T CELL ACTIVATION, EXHAUSTION AND CYTOKINE PRODUCTION **Code** 15

Authors Names Ernesto Marcos Lopez (1)

Authors Affiliations 1. Standard BioTools

Topic Hematology

Abstract

Introduction CAR T cell therapy has emerged as a groundbreaking advancement in cancer treatment, offering new hope for patients with previously intractable forms of the disease. CAR T cell therapy involves the engineering of patients' T cells to target and eliminate cancer cells, a concept that has transformed the landscape of oncological therapeutics. Despite its promise, the effectiveness of CAR T cell therapy is intricately linked to the complex interplay between engineered T cells and the tumor microenvironment. In particular, CAR T therapy targeting solid tumors has shown limited efficacy due to various factors such as reduced trafficking, an immunosuppressive tumor milieu, reduced persistence and antigen escape. Thus, a better understanding of CAR T biology to maximize therapeutic outcomes while minimizing off-target effects – such as cytokine release syndrome and neurotoxicity – is necessary to overcome these limitations.

Methods Cell culture. Anti-CD19 CAR T cells (BPS Bioscience) were activated with ImmunoCult Human CD3/CD28/CD2 T Cell Activator (STEMCELL Technologies) and IL-2 (10 ng/mL) in ImmunoCult-XF T Cell Expansion Medium for two days, then passaged for an additional five days with IL-2. Cryopreserved PBMC (STEMCELL) and NALM6 cells (ATCC) were thawed and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin. Panel design. The Maxpar Direct Immune Profiling Assay was supplemented with antibodies from the Maxpar Direct Basic Activation Expansion Panel (IL-2, IFNγ, TNFα, granzyme B, perforin and CD107a), five antibodies from the T Cell Expansion Panel 3 (OX40, TIGIT, CD69, TIM-3 and 4-1BB) and PD-1. Channel 165Ho was used for CAR CD19 detection, using either a directly conjugated or biotinylated G4S Linker antibody (Cell Signaling Technology) or biotinylated CD19 CAR Detection Reagent (Miltenyi) and streptavidin-165Ho as a secondary reagent. Samples were acquired on a CyTOF™ XT instrument in batch acquisition mode.

Results Optimal CAR T detection We first compared CAR T detection options for sensitivity and specificity using a CD19 CAR-transduced cell line spiked into healthy donor PBMC. Indirect staining was compared against a directly conjugated or biotinylated anti-G4S E702V Linker antibody from Cell Signaling Technology. Indirect staining with the Miltenyi CD19 CAR Detection Reagent yielded the best resolution and lowest background. The anti-G4S Linker antibody demonstrated higher signal and reduced nonspecific staining when directly conjugated to 165Ho (using a Maxpar X8 Antibody Labeling Kit) compared with indirect staining. About 50% of the CD19 CAR T cell line lacked CAR-CD19receptor expression, necessitating pre-labeling of the cell line with CD45 conjugated to 115In. However, CD45-115In was not required for dimensionality reduction and FlowSOM clustering as most (about 87%) of the CAR T cells clustered together based on their distinct expression profile of the full panel of markers (Figure 3). Phenotypic and functional characterization of CAR T cells To simulate post-infusion immune profiling of CAR T treated patients, unstimulated or NALM6-activated CAR T cells were spiked into healthy donor PBMC at 1:1, 2:1, 5:1 and 10:1 (PBMC:CAR T) ratios. This admixture was then stained with the full 43-marker panel and evaluated for lineage marker expression. The CAR T cells were easily resolved from the PBMC background even at 10-fold lower frequency. UMAP projection using the full panel also easily grouped the majority of CAR T cells into a distinct region, while FlowSOM clustering led to 11 out of 30 metaclusters highly enriched for CAR T cells. Maxpar Pathsetter for the automatedquantitation and characterization of CAR T cells Maxpar Pathsetter is an automated and complete analysis solution for samples stained with the Maxpar Direct Immune Profiling Assay and Expansion Panels.

Conclusion Optimal CAR T detection and phenotyping was achieved with this comprehensive mass cytometry panel of 43 markers, ideal for immune profiling CAR T cells at all stages of CAR T therapy. CAR T cells spiked into healthy donor peripheral blood mononuclear cells – to simulate immune profiling after infusion into patients– could be clearly resolved and immunoprofiled. CAR T detection can be tailored to each CAR T product by combining a CAR T receptor detection reagent with a secondary antibody or probe conjugated to a metal tag. The pre-optimized and validated Maxpar™ Direct Immune Profiling Assay has been used extensively for multi-site and longitudinal immune profiling studies due to its ease of use and reproducibility. The lyophilized single-tube Maxpar Direct Immune Profiling Assay and Expansion Panels are optimized to work together while still allowing for expansion of the panel (for example, to incorporate sample barcoding for batched acquisitions).

Conflict of Interest Yes. Standard BioTools.

Title DEVELOPMENT OF AN INNOVATIVE REAGENT FOR A STANDARIZED ANTI-CD19 CAR-T CELLS DETECTION BY FLOW CYTOMETRY **Code** 40

Authors Names Eduarda Da Silva Barbosa (1,2); María Herrero-García (2-5); Ricardo Jara-Acevedo (6); César Vega-Llusiá (6), Sara Gutiérrez-Herrero (2-5), Lourdes Martín-Martín (2-5,7); Alejandro Romero-Casañas (6); Lucía López-Corral (2-5); Alejandro Martín García-Sancho (4,5,7,8); Ana-África Martín-López (4,5,7,8); Estefanía Pérez-López (4,5,7,8); Alberto Orfao (2-5,7)

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Topic Hematology,Biotechnology and new applications

Abstract

Introduction Chimeric antigen receptor T-cell (CAR-T) therapy has emerged as a groundbreaking approach for cancer treatment. This immunotherapy is especially well-established for relapsed/refractory B-cell malignancies but despite its initially promising results, the side effects after CAR-T cell infusion remain a major challenge and more than 50% of treated patients are non-responders or further relapse. So far, many studies have pointed out that specific features of the product as well as the magnitude of the in vivo expansion, persistence and functional circulating CAR-T cells have an impact in the response to treatment. Thus, a standardized monitoring approach which allows to quantify the in vivo CAR-T cells together with their characterization remains a critical unmet need. In this regard flow cytometry (FCM) could make a difference with PCR because despite its theoretically lower sensitivity, FCM is an accessible technique which brings quick results and allows simultaneous quantification/characterization of CAR-T and residual immune cells. However, to efficiently monitor CAR-T cells using FCM, it is essential to employ detection reagents that optimize antigen accessibility, distribution and stability. High epitope accessibility enhances interaction with the CAR receptor, while a homogeneous distribution on the cell surface minimizes non-specific adsorption and ensures a uniform signal. The multivalency and high epitope density of the reagents promote more stable binding, leading to increased signal. Reducing background noise improves the signal-to-noise ratio, enabling more precise detection and fluorochrome stability extends signal duration and intensity, ensuring more reproducible measurements.

Methods In this context our aim is to develop and validate a standardized and clinically broader applicable reagent for anti CD19 CAR-T detection suitable for both commercial and academic products in patients with different B-cell malignancies. To this end, a battery of reagents generated through an advanced conjugation strategy for recombinant proteins has been developed and validated. During the first validation rounds we tested up to 50 candidate CD19 proteins in more than 90 fresh peripheral blood (PB) samples of patients with different B-cell malignancies treated with either commercial or academic CAR-T cells on which CAR-T cell presence was previously confirmed with a commercial reagent of reference. In each validation round the selected PB sample was stained with 4 Mabs (to a proper identification T and NK cells) plus the CD19p. In all of them, the candidate CD19p were compared with different commercial reagents. Stained samples were treated according to a FacsL protocol and then measured on a spectral cytometer. Comparisons between all the reagents were based on % of detectable CAR-T cells within total T-cells, median fluorescence intensity (MFI) and Stain Index (SI) of CAR-T cells. The comparison of all these parameters brought as candidate on which we performed extra analysis to validate: the inter-lot reproducibility, its stability over time and its suitability to different staining protocols.

Results As a result of all our assays we confirmed that our reagent optimizes protein presentation, allowing for lower reagent titers while significantly reducing non-specific signals. It perfectly discriminates CAR-T cells from normal T cells showing a median (range) SI of 94.1(35.8-211.2) in a short single-step FacsL protocol. This resolution is sustained independently of the reagent lot considered and also independently of the staining procedure used and is 9 times higher than the resolution showed by the commercially available competitor used for one-step staining procedures and 1.4 times higher than the resolution showed when using a widely distributed commercial reagent in combination with a secondary antibody.

Conclusion In summary, we developed and validated a new anti CD19 CAR-T cell detection reagent suitable for different staining procedures and samples, allowing not only shorter protocols but also revealing higher resolution rates compared to commercially available proteins.

Conflict of Interest Yes. RJA, CVL and ARC are employed by Immunostep SL.

Title CHILDREN ALL DIAGNOSE AND FOLLOW-UP ACCORDING TO ALLTOGETHER PROTOCOL: IPO PORTO EXPERIENCE **Code** 65

Authors Names Bruno Fernandes (1); José Pereira (1); Paulo Silva (1); Andreia Pinto (1); Carina Faria (1); Patrícia Maia (1); Carla Azevedo (1); Catarina Fonseca (1); Julieta Silva (1); M Emília Sousa (1); Carlos Palmeira (1,2,3); Gabriela Martins (1)

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Topic Hematology

Abstract

Introduction The aim of the Alltogether clinical trial is the study of acute lymphoblastic leukemia in children and young adults. The main goal is to improve survival and reduce side effects, as well as learn more about the disease to further improve treatment. Flow cytometry has a predominant and growing role in the diagnosis and follow-up of ALL, particularly through the methodologies included in the so-called next-generation flow (NGF). We will discuss the role of flow cytometry in diagnosis and in measurable residual disease (MRD) testing, as well as show some data regarding IPO Porto experience on this matter.

Methods This review included 79 cases of ALL pediatric patients enrolled in the AllTogether clinical trial, assessed by NGF consecutively between 2021 and 2024 in the Clinical Pathology Service Cytometry Lab of IPO-Porto. The immunophenotypical evaluation of the bone marrow aspirate samples at different time points was carried out in accordance with the clinical trial guidelines and Euroflow/ALLTogether panels. The evaluation of MRD by next-generation sequence (NGS) was obtained by consulting the medical records.

Results In the 79 cases included, immunophenotyping revealed 70 B-ALL and 9 T-ALL cases. NGF MRD was performed in 176 samples with a median LOD 0.0002% and a median LLOQ 0.001%. 51 samples were positive. Regarding the MRD evaluation by NGF vs NGS we found that 39 positive samples and 111 negative samples were concordant. 12 samples were positive by NGS and negative by NGF. Meanwhile, 14 samples were positive only by NGF. In cases of positive MRD for both methods, NGF detected a higher number of disease in 71.8% of cases.

Conclusion According to the experience of this laboratory, the use of standardized protocols and international guidelines, such as Euroflow and AllTogether, allows for the adequate characterization of ALL in a pediatric population. NGF proved to be a sensitive method for detecting MRD in these samples, with detection limits between 10^5 and 10^6. A larger number of cases and longer follow-up time will provide more data that can be used to optimize these methodologies, contributing to a better MRD assessment and improving the prognosis of these patients.

Conflict of Interest No.

Abstracts

ePoster

Title OPTIMIZING TIL EXPANSION FOR CANCER IMMUNOTHERAPY: A PROTOCOL INTEGRATING IL-2, IL-7, AND IL-15. **Code** 2

Authors Names Daniel Tovar Manzano (1); Nabil Subhi-issa Marin (1); Alejandro Pereiro-Rodríguez (2); María Guzmán Fulgencio (2); Miguel Fernández-Arquero (2); Pedro Pérez Segura (2); Alberto Ocaña Fernández (2); Silvia Sánchez-Ramón(2)

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Topic Immunology

Abstract

Introduction Introduction Effective T-cell expansion is a critical step in adoptive cell therapies and poses a challenge for cell transfer in immunotherapy. This study aimed to optimize TIL expansion by evaluating the effectiveness of a protocol using IL-2, IL-7, and IL-15 in TILs derived from two lung adenocarcinomas and one melanoma tumor. The impact of the protocol on T-cell activation, exhaustion, and functionality was assessed.

Methods TILs were expanded ex vivo over 14 days using a protocol incorporating IL-2 (10 U/mL), IL-7 (1800 U/mL), and IL-15 (6 U/mL). The phenotype of T cells was analyzed by multiparametric flow cytometry at three points: baseline, Pre-REP, and REP. Descriptive values and qualitative assessments were used to compare cellular subsets of interest.

Results Expansion in vitro of TILs from different tumor samples using our protocol of IL-2, IL-7, and IL-15 revealed significant expression of activation and functionality markers in TILs. Exhaustion markers, such as PD-1, were decreased in CD8+ cells, decreasing from an initial range of 87.75%-69.20% to a final range of 34.88%-22.40%. Regarding activation markers, elevated expression of 4-1BB was observed in CD8+ T cells at the end of expansion, reaching values between 69.32% and 99.82%. The CD28 marker maintained high expression in CD4+ cells throughout the process, with final values between 81.30% and 95.30%, while CD28 expression in CD8+ cells progressively increased to reach a range of 72.60%-84.90%, indicating sustained activation. Moreover, cell viability at the end of the protocol exceeded 93%, reflecting minimal cell death expansion process. Additionally, over 93% of the cells were CD3+, emphasizing the high purity of the expanded T-cell population. Among these CD3+ cells, approx

Conclusion Our optimized expansion protocol effectively expands TILs, enhances their activation and functionality, and reduces exhaustion markers. These results are promising for adoptive cancer immunotherapy. Current efforts focus on validating these findings to understand the dynamics of activated T-cell proliferation under different protocols and identify optimal conditions for ex vivo expansion.

Conflict of Interest No.

Title BEYOND INFECTIONS: THE VALUE OF THF1 CELLS IN IDENTIFYING HIGH-RISK PATIENTS WITH COMMON VARIABLE IMMUNODEFICIENCY **Code** 3

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Topic Immunology

Abstract

Introduction Common variable immunodeficiency (CVID) is the most common symptomatic primary immunodeficiency and has a high clinical heterogeneity. Up to 60% of patients develop non-infectious complications such as autoimmune cytopenias, interstitial lung disease or lymphoproliferation, which are associated with increased morbidity and mortality. This study explores the association between T helper follicular cells type 1 (THF1) and this subgroup of patients.

Methods Determination of different T and B lymphocyte subpopulations in peripheral blood by flow cytometry was performed in 41 patients with CVID and 20 healthy controls (HC). Clinical data were collected and the cohort of patients was stratified into two groups: Non-Infectious Complications (NIC) and No Complications (NC).

Results The results show that the THF1 subpopulation can identify patients who develop autoimmunity and/or granulomas, with an area under the curve of 0.82. Regarding the group analysis, a marked absolute decrease is observed in the main lymphocyte subpopulations, including CD4, CD8, and NK lymphocytes, in the NIC group compared to the NC and HC groups. In the CD4 compartment, the NIC group presents a significant reduction in TH17, TH2, and regulatory T lymphocytes, as well as a marked increase in CD21low B cells compared to the NC and HC groups. Serum free kappa light chains and immunoglobulin A are also decreased in the NIC group compared to the other groups.

Conclusion THF1 cells emerge as a useful biomarker for stratifying high-risk CVID patients, allowing for closer monitoring. We suspect that the decrease in regulatory T cells and the expansion of CD21low B cells, along with THF1 cells, are involved in the pathophysiology, making them potential therapeutic targets. However, functional studies are needed to validate this hypothesis.

Conflict of Interest No.

Title IMPACT OF COMBINED AND PREDOMINANTLY-ANTIBODY IMMUNODEFICIENCIES IN THE INNATE COMPARTMENT **Code** 4

Authors Names Maurice O´Gormann (1); Martin Perez-Andres (2,3,4); Quentin Lecrevisse (2,3,4); Mirjam van der Burg (5); Tomas Kalina (6); Carolien Bonroy (7,8); Susana L. Silva (9,10,11,12); Eduardo Lopez-Granados (13); Cristina Serrano (14); Alba Torres-Valle (2,3,4); Sonia Arriba-Mendez (15); Ana E. Sousa (9,10,11); Mattias Hofmans (7,8); Jacques J.M. van Dongen (2,3,16,17); Alberto Orfao (2,3,4)

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Topic Immunology

Abstract

Introduction Genes traditionally associated to combined immune deficiencies (CID), and predominantly antibody deficiencies (PAD) might result in abnormalities outside the lymphoid compartment. However, the role of these genes in the innate compartment has been poorly explored.

Methods Blood counts of neutrophils, eosinophils, basophils, classical and non-classical monocytes (cMo and ncMO), and dendritic cells (DC) were evaluated in 22 severe (8 IL2RG, 3 JAK3 and 11 RAG) and 32 other CID (4 ZAP70, 7 CD40L, 7 WAS, 7 DiGeorge, 7 ATM), and 19 PAD (13 BTK, 6 PI3KCD) patients, in parallel to 253 age-matched healthy donors, using EuroFlow methods.

Results Significantly ($p<0.05$) reduced blood counts of eosinophils, basophils, cMo, ncMo and DC were observed in IL2RG patients vs. age-matched healthy donors (41±84 vs. 235±180 cells/uL, 9±21 vs. 37±20 cells/uL, 508±279 vs. 1,015±560 cells/uL, 39±44 vs. 187±126 cells/uL 7±7 vs. 25±14 cells/uL, respectively). Expanded eosinophils (3212±4527 vs. 312±230 cells/uL) and neutrophils (7486±8450 vs. 3273±1998 cells/uL) were the only myeloid alterations observed in JAK3 and RAG patients, respectively. In blood from CD40L patients, significantly reduced counts of cMo (340±141 vs. 657±312 cells/uL) and ncMo (37±30 vs. 89±95 cells/uL), together with DC (6±4 vs. 23±14 cells/uL) were observed. In contrast, no altered myeloid subsets were observed in other non-severe CID (ZAP70, WAS, Digeorge), except for increased counts of ncMo in ATM patients (197±160 vs. 69±63 cells/uL). Increased blood counts of neutrophils (5315±2789 vs. 3649±1378 cells/uL and cMo (861±342 vs. 627±289 cells/uL) were observed in BTK pa

Conclusion In addition to well characterized defects in the lymphoid compartment, unique profiles of myeloid defects were observed in association with IL2RG, CD40L, and PI3KCD pathogenic mutations. These results emphasize the role of these signaling molecules also in the innate compartment.

Conflict of Interest No.

Title FLAER AS A NEW DIAGNOSTIC MARKER OF ACUTE PROMYELOCYTIC LEUKEMIA **Code** 6

Authors Names María Beatriz Álvarez Flores (1); María Sopeña Corvinos (2); Raquel Guillén Santos (2); Fernando Cava Valenciano (2)

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Topic Hematology

Abstract

Introduction FLAER is widely used in the diagnosis of PNH by flow cytometry because it binds directly to the GPI anchor on the cell membrane, rather than to GPI-dependent proteins.
This study examined the expression of FLAER in both lymphoid and myeloid precursors from the bone marrow of control subjects, as well as in lymphoid and myeloid precursors from various acute myeloid leukemias (AML) and B-cell acute lymphoblastic leukemia (B-ALL). A significant reduction in FLAER expression was observed specifically in Acute Promyelocytic Leukemia (APL).

Methods This study included 71 samples from patients with hematologic malignancies: 53 AML cases (14 APL), 7 B-ALL, 3 blastic plasmacytoid dendritic cell neoplasm, and 8 myelodysplastic syndromes. 16 control samples were collected from bone marrow aspirates (12 ITP, 4 B-NHL with no bone marrow involvement). Flow cytometry was performed using a BD FACSCanto II™ cytometer, and data were analyzed with Infinicyt™ software. To ensure MFI comparability over time, PMT voltages were initially adjusted using unlabeled, lysed whole blood cells to optimize dim cell resolution. These target values were used for subsequent calibrations to maintain standardization. Additionally, CD13/CD33 expression analysis helped differentiate APL from other AML cases with an APL-like phenotype and assess distinct expression profiles for accurate leukemia classification.

Results APL promyelocytes showed significantly lower FLAER binding (MFI) compared to other AMLs and MDS. Except for AMLs with a monocytoid component (M4–M5), AML samples exhibited lower FLAER intensity than control bone marrow precursors. Although FLAER binding was lower in FAB M0-M1 than in FAB M2, this difference was not statistically significant, possibly due to the small number of M2 samples. In cases where pathological precursors were APL-like phenotype and had low FLAER MFI, CD13/CD33 expression pattern analysis identified all APL cases (100%). A comparison of FLAER signal, expressed as relative differences, revealed that no AML samples showed greater FLAER binding than control myeloid precursors. The weakest binding was seen in APL samples (0.18 of the control signal), followed by M2 (0.37), M0–M1 (0.49), AML sec (0.66), MDS (0.88), and M4–M5 (0.93). The study of MRD in APL also revealed a lower expression of FLAER when the presence of disease was detected after treatment.

Conclusion The integration of FLAER into the AML study panel could prove highly valuable for the diagnosis of APL, even in AML cases exhibiting an APL-like phenotype, by assessing the CD13/CD33 expression pattern. Moreover, FLAER analysis played a significant role in the investigation of measurable residual disease in APL, further enhancing its diagnostic and monitoring potential. FLAER is already widely available in most clinical flow cytometry laboratories, making its implementation very straightforward.

Conflict of Interest No.

Title NOVEL IMMUNOPHENOTYPIC STRATEGIES FOR IMPROVED DIAGNOSTIC CLASSIFICATION OF MATURE T-CELL NEOPLASMS **Code** 7

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Topic Hematology

Abstract

Introduction T-cell chronic lymphoproliferative disorders (T-CLPD) are a heterogeneous group of mature T-cell neoplasms, usually with aggressive behavior. The WHO/ICC-2022 classifications include >25 categories, but diagnosis remains challenging due to overlapping biological features among and within categories, and similarities between clonal and reactive T cells. Treatment is complex, and as blood immune profiles in T-CLPD remain poorly understood, patients often cannot benefit from immunotherapy. Our goal was to define the immunophenotypic patterns of tumor cells in each diagnostic category (based on their similarities with normal T-cell subsets), and identify unique immune profiles associated with specific T-CLPD entities, to ultimately refine diagnosis.

Methods A total of 73 samples from 3 subtypes of T-CLPD –T-prolymphocytic leukemia (T-PLL, n=18), Sezary syndrome/mycosis fungoides (SS/MF, n=18), and T-large granular lymphocytic leukemia (T-LGLL, n=37) – were analyzed by flow cytometry, using EuroFlow panels and SOPs (www.euroflow.org) and the Infinicyt software; 116 age- and sex-matched adult healthy donors were also included. Tumor cells were classified into maturation and functional (Th) compartments based on their phenotypic similarities with normal T cells; in parallel, we analyzed in each category the residual immune system in blood.

Results We identified predominant phenotypic profiles and distinct immune alterations per category. T-PLL exhibited a predominant naive-central memory (N/CM) phenotype, characterized by high Th heterogeneity and profound immune impairment. This was marked by increased blood counts of monocytes, dendritic cells (DCs), B cells, NK cells and ILCs (mainly ILC3), alongside with a reduction in normal T cells. Patients with more advanced tumor differentiation showed higher frequency of anemia, and a trend toward lower overall and progression-free survival. Additionally, those whose tumor cells presented ≥2 Th phenotypes had higher rates of splenomegaly, thrombocytopenia, tumor cell burden and a trend toward higher pleural/peritoneal effusion and white blood cell counts. Likely, disease progression correlated with elevated monocyte, NK-cell, and ILC2 counts. SS/MF cells were primarily classified in memory stages (MF slightly more mature) with either Th2 or Th17-restricted phenotypes. The immune system also showed significant alterations, including neutrophilia and decreased counts of DCs, NK cells and cytotoxic T cells, potentially reflecting their increased migration from blood to the skin. In contrast, T-LGLL cells were predominantly terminal effector cells with a functional profile exclusively restricted to Th1 cells (even CD183- due to activation). T-LGLL patients showed the mildest immune impairment, varying by the clonal T-cell lineage and presence of STAT3 mutations, these latter patients showing pronounced reductions in neutrophils, DCs, and NK cells, as well as a Th1 $CD183^-$ phenotype, which might serve as a surrogate marker for STAT3 mutations in $T\alpha\beta CD8^+$ cases. Further analysis of normal T-cell subsets revealed a reduction (p<lt;0.05) in the earliest T-cell maturation stages (N/CM) in T-PLL and SS/MF, with only a minor reduction in N/CM T cells in T-LGLL. This finding suggests a possible shift in (thymic) production from normal to tumor-associated (e.g., naive) T-cells.

Conclusion Overall, we show that Th-related phenotypic heterogeneity progressively narrows from less mature to more differentiated tumor T-cell subtypes. We demonstrated blood alterations affecting both innate and adaptive immunity, with category-dependent variability. Finally, we highlight that the Th1 $CD183^-$ phenotype of tumor cells or reduced neutrophils, DCs, and NK cells counts could serve as a surrogate marker for STAT3 mutations in $T\alpha\beta CD8^+$ LGLL. The unique maturation and Th-related patterns, together with the immune profile, could aid in stratifying T-CLPD, including for disease outcome (e.g., in T-PLL).

Conflict of Interest No.

Title ALTERED B-CELL, PLASMA CELL AND ANTIBODY IMMUNE PROFILES IN BLOOD OF SYSTEMIC MASTOCYTOSIS **Code** 8

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Topic Hematology

Abstract

Introduction Systemic mastocytosis (SM) is a heterogeneous disease characterised by an expansion of morphologically and immunophenotypically aberrant mast cells (MC) which in the great majority of cases (>95%) exhibit an activating mutation of the KIT gene that leads to their constitutive activation, and with that, to the broad release of MC mediators that might act on the tumour microenvironment including other immune cells. Here we investigated the blood distribution of B-cell, plasma cell (PC) and antibody-isotype compartments in SM patients to deepen our understanding of the role that the humoral response may play in the clinical and biological behavior of the disease.

Methods We used spectral flow cytometry and the EuroFlow Immunomonitoring panel and Lymphocyte Screening Tube to quantify B-cells, PC and their subsets in blood of 108 SM patients - 35 bone marrow mastocytosis (BMM), 64 indolent SM (ISM), 9 aggressive SM (ASM) together with paired bone marrow (BM) samples of 31/108 SM patients. To normalize the distribution data of blood immune cells, 108 age-matched healthy donors (HD) were used. In parallel, immunoglobulin (Ig) M, IgD, IgG, IgA and IgE plasma levels of were measured.

Results Compared to HD, SM patients showed an increased immature B-cell production in BM (P=0.003) associated with a greater release of pre-germinal center immature (P<0.001) and naive CD5+ B-lymphocytes (P<0.001) to blood, but a pronounced decrease in PC counts of all different IgH-isotypes and subclasses (P≤0.001) with maintained normal Ig plasma values. However, although the total amount of Igs in plasma was within the normal range, a more detailed analysis by isotype showed altered Ig levels among the distinct subtypes of the disease, including an increase in IgM (specifically among ISM and ASM patients) and IgD (at the expense of patients with BMM and ISM) in plasma (P<0.027), together with abnormally low levels of IgG (P=0.008) along with significantly increased levels of IgE in BMM cases (P<0.001). Of note, using multivariate analysis, different immune profiles were found per diagnostic subtype of the disease with progressively greater counts in blood of immature B-lymphocytes together with decreased IgMD+, IgG2+, IgA1+ and IgA2+ MBC (P≤0.032) and elevated IgM (P=0.017) plasma levels in ASM cases, increased IgM (P=0.001) and IgD (P=0.001) plasma levels in ISM patients and exacerbated IgE (P<0.001) with decreased IgG (P=0.008) plasma levels in BMM cases. Moreover, we observed an association between the altered distribution of some B-cells subsets and the clinical behavior of the disease, specifically, the increase of immature B lymphocyte count in blood reflected in a higher frequency of osteoporosis, organomegaly (hepatomegaly and lymphadenopathy) and a history of Helicobacter pylori infection (P<0.024) as well as the decrease in different blood PC subpopulations was associated with a higher prevalence of mast cell mediator release symptoms (flushing or pruritus) (P<0.044), and lastly, the existence of increased plasma IgE values correlated with a higher frequency of anaphylaxis and insect venom allergy, and a lower prevalence of bone sclerosis (P<0.005).

Conclusion Our results reveal a significant dysregulation of the B-cell and PC compartments in blood of SM patients and point out a potential creased B-cell production and migration to tissues, associated with lower PC counts in blood but increased IgM, IgD or IgE antibody plasma levels leading to uniquely altered humoral immune profiles in BMM vs ISM vs ASM patients.

Conflict of Interest No.

Title FLXFLOW-HDSPECT: BUILDING A COLLABORATIVE INFRASTRUCTURE FOR ADVANCED SPECTRAL FLOW CYTOMETRY IN PORTUGAL **Code** 9

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Topic Biotechnology and new applications

Abstract

Introduction 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.

Methods 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.

Results The FLxFlow-HDSpect infrastructure has been integrated into the existing FLxFlow network (https: flxflow.pt/), 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.

Conclusion 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 at both national and international levels. This collaborative approach is advancing biomedical research, opening new possibilities for understanding diseases and cellular mechanisms.

Conflict of Interest No.

Title PREVALENCE, PHENOTYPE AND MOLECULAR PROFILING OF CIRCULATING T-CELL CLONES OF UNCERTAIN SIGNIFICANCE IN HEALTHY SUBJECTS ASSESSED BY HIGH-SENSITIVE FLOW-CYTOMETRY **Code** 11

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Topic Hematology

Abstract

Introduction T- cell clones of uncertain significance (T-CUS) indicates the presence of persistent (>6m) circulating monoclonal T cells in blood, in otherwise healthy donors (HD), i.e., in the absence of symptoms or signs of lymphoproliferative disorder. It has been postulated that T-CUS would represent a premalignant condition, but its clinical significance remains undetermined so far. Small T-cell clones are being increasingly detected in blood, due to the recent availability of new immunophenotypic-based methods in routine laboratories; however, its prevalence (and in-depth characterization) in a population-based screening context is still unknown. In this study we analysed the presence of clonal T-lymphocyte populations, to establish the real prevalence of T-CUS, and the phenotypic and molecular profiling of the T-cell clones identified in blood in the general population >18 years of Salamanca, Spain.

Methods Peripheral blood (PB) samples from 1,254 HD, 861 men (69%) and 393 (31%) women, were analyzed using high-sensitivity flow cytometry with the EuroFlow lymphoid-screening tube (LST) plus anti-TRBC1 (which recognizes one of the two mutually exclusive T-cell receptor β-chain constant domains) to assess T-cell clonality. Extended panels were used for further characterization, in case of identification of T-cell clones. All samples were processed following the EuroFlow Consortium SOPs, and analyzed on a conventional FACS Canto II cytometer. Data-analysis was performed using INFINICYTTM. After sample processing and analysis, the aberrant and potential clonal populations were purified by FACSorting, to evaluate the TCR rearrangement by PCR to confirm T-cell clonality. Somatic STAT3/5b mutations were assessed in each suspected/clonal cell population (previously purified) by PCR.

Results T-CUS were detected in 260/1,254 subjects (21%) (19% in men and 24% in women; p>0.05), of which 33% were found to additionally carry B-cell clones (MBL) in blood. The frequency of T-CUS increased with age: 11% (18-30 years), 19% (31-40), 16% (41-50), 19% (51-60), 36% (61-70), and 71% (>71 years) (p0.05) between monoclonal and bi-multiclonal cases; also, no age differences were found between mono and bi-multiclonal cases. The most reliable phenotypic profile for detection of T-CUS included CD56 positivity with monomodal expression of TRBC1 within Tαβ expansions, particularly together with CD5 loss; in turn, Tγδ clones were identified by downregulation of CD3/TCRγδ and strong CD8 expression. According to the T-cell lineage, 51% of the 412 T-CUS populations corresponded to TαβCD8+CD4-, 32% to TαβCD4+CD8-/lo, 8% to Tγδ, 5% to TαβCD4-CD8-/lo and 4% to TαβCD4+CD8+ cell expansions. Most (90%) populations showed a phenotype resembling large granular lymphocytes (LGL), characterized by intracellular expression of granzyme B and a usually terminal effector maturation stage, independently of the cell lineage. No STAT3/5b mutations were detected in any of the 412 T-cell clones.

Conclusion With a high-sensitive flow-cytometry approach, including anti-TRBC1, we found a high prevalence of T-CUS (21%) in adult HD from the general population, with either mono or multiclonality (56% monoclonal vs 44% bi-multiclonal). The frequency slightly increases between 18-60 years of age, and thereafter dramatically increases, particularly in subjects >70y-old, without differences by sex. Most T-CUS populations correspond to TαβCD8+CD4-lineage, being the majority (90%) compatible with a T-LGL phenotype, and lack STAT3/5b mutations.

Conflict of Interest No.

Title DECODING GUT'S IMMUNE SIGNATURE: UNSUPERVISED HIGH-DIMENSIONAL CHARACTERIZATION OF ILEAL AND COLONIC LANDSCAPES IN HEALTH AND IBD **Code** 12

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Topic Immunology

Abstract

Introduction Inflammatory bowel disease (IBD), including Crohn´s disease (CD) and ulcerative colitis (UC), is a chronic disorder of unknown aetiology that involves a pathological response of the immune system resulting in chronic inflammation of the gastrointestinal tract. Nevertheless, the composition of the specific immune infiltrate in the human intestine remains partially unknown due to the technical difficulties that its characterization entails. In this regard, spectral cytometry offers a powerful and high-dimensional approach that enables the simultaneous analysis of complex panels (>40) hence allowing the identification of several immune cell subsets—including rare or exiguous ones—that conventional flow cytometry might miss. Hence, we hereby aimed to perform an unbiased characterization of the human intestinal immune fingerprint in health and IBD in order to get a deeper insight into the specific immune mechanisms underlaying CD and UC pathogenesis.

Methods Biopsies from the ileum and colon were obtained from non-inflamed controls. In addition, colonic samples from patients with active UC (aUC), as well as ileal samples from CD patients, both active (aCD) and quiescent (qCD) were obtained. In all cases, biopsies were cryopreserved until lamina propria mononuclear cells (LPMC) were obtained. Cells were further stained with a 40-plex panel and acquired on a 5-laser spectral cytometer. Unsupervised analyses (including dimensional reduction and clustering methods) were performed using the OMIQ® platform. Cluster identification was performed based on markers expression and subsequent hierarchical gating approaches were applied to validate the obtained results.

Results Unsupervised analyses allowed us to identify a total of 56 different cell clusters within the human intestinal immune infiltrate. Following subsequent hierarchical validation approaches it was found that, in health, the colon carried higher levels of IgA+ plasmablasts. Although there were 14 clusters differentially expressed in the colon from aUC patients (with an expansion of effector memory T-cells), that however could not be validated by classical gating approaches. As for the ileum, CD patients displayed a specific reduction of memory B-cells, irrespectively of disease status -active/quiescent-, which was more prominent in the latter. Last, CD8+ effector T-cells were expanded in the ileum from qCD patients referred to those with active disease.

Conclusion We hereby have performed an unbiased characterization of the human intestinal immune infiltrate in the colon and ileum from controls revealing the presence of regional immune differences through the human gastrointestinal tract in health at the time that we have also unveiled specific immune differences between CD and UC patients. This unbiased exploration therefore enhances our understanding of cellular diversity in complex tissues like the GI-tract contributing to understand the physiological mechanisms of IBD.

Conflict of Interest No. This work has been funded by the GETECCU 2022 grant.

Title SPECTRAL AND COMPUTATIONAL CYTOMETRY IDENTIFIES CIRCULATING NKT-LIKE CELL SUBSETS AT HOSPITAL ADMISSION AS NOVEL BIOMARKERS TO PREDICT DISEASE PROGNOSIS IN ACUTE PANCREATITIS. **Code** 13

Authors Names Carolina G de Castro (1); Paloma Cal-Sabater (1); Elisa Arribas-Rodríguez (1); Álvaro Martín-Muñoz (2); Alejandro G. del Hierro (1); Marina Perez Mazzali (1); Jessica Matesanz Isabel (3); Hugo Gonzalo Benito (3,5,6); Luis Fernández-Salazar (4); Lourdes Ruiz Rebollo (4); David Bernardo (1,6); Sara Cuesta-Sancho (1)

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Topic Immunology

Abstract

Introduction Acute pancreatitis (AP) is one of the most common diseases underlying gastroenterology hospital admissions in Western countries. While most cases are mild, about 20–30% of patients develop severe complications, including organ failure with potentially devastating consequences. Although an early identification of patients with subsequent severe complications is essential, current scoring systems have shown limited predictive value. Given therefore that the immune system mediates AP severity, we hereby aimed to characterize the circulating immunome of AP patients at hospital admission in order to identify novel biomarkers that can subsequently predict disease prognosis and/or identify novel targets to perform a precision medicine.

Methods Peripheral blood mononuclear cells (PBMC) at hospital admission from 40 patients with AP were obtained. Patients were further divided into those with subsequent mild (n=20) or severe (n=20) disease evolution and referred to PBMC from 20 sex and age-matched non-inflamed controls. Cells were stained with a 40-plex panel and acquired on a spectral cytometer. Results were analysed using unsupervised clustering and dimensional reduction algorithms. Clusters differentially expressed between patients with mild and severe AP were further validated by hierarchical gating approaches, while their function was interrogated following ex-vivo stimulation.

Results UMAP and FlowSOM approaches identified a total of 120 immune cell clusters which were further defined based on their phenotype. Although AP patients displayed a differential immune signature referred to controls, further analysis at hospital admission identified 3 clusters of immune cells differently expressed among those with subsequent mild or severe disease evolution. Indeed, hierarchical gating validation revealed that, at hospital admission, patients with subsequent severe AP had reduced levels of circulating CD2+CD8dim NKT-like cells referred to those who had a better disease outcome. Moreover, these cells displayed a more cytotoxic profile following ex-vivo stimulation in patients with severe AP, hence also confirming immune functional differences based on subsequent AP prognosis.

Conclusion Our results have revealed circulating NKT-like cells as novel prognosis biomarkers in AP, hence allowing an early identification of patients with subsequent severe outcome. Moreover, the identification of a more cytotoxic function of these cells in patients with severe AP may provide novel tools to identify the specific mechanisms underlying AP progression. Altogether these findings highlight the potential of NKT-like cells as predictive biomarkers of AP severity and possible targets for therapeutic intervention.

Conflict of Interest No.

Title FAST AND CONVENIENT HUMAN IMMUNE PROFILING OF FRESH AND LONG-TERM STABILIZED WHOLE BLOOD **Code** 14

Authors Names Ernesto Marcos Lopez (1)

Authors Affiliations 1. Standard BioTools

Topic Immunology

Abstract

Introduction The CyTOF advantage, overcoming challenges of fluorescence technology CyTOF® flow cytometry uses metal-tagged antibodies to identify cellular and functional phenotypes. CyTOF technology overcomes major hurdles of fluorescence-based cytometry and provides a streamlined and flexible workflow in clinical research. Challenges and solutions to whole blood immune profiling. Accurate phenotyping of immune cells in whole blood (WB) from patients with cancer is critical for making disease prognoses and monitoring clinical efficacy of immunotherapies. Fresh WB must be analyzed within 24 hours of collection to minimize changes in cellular composition. However, WB collection and cytometric analysis are often performed at different sites, which can result in sample processing delays. Several WB preservation reagents have been developed to address this challenge, including PROT1 (Smart Tube Inc.) and Cytodelics Whole Blood Cell Stabiliser (Cytodelics). Yet not all antibody panels are compatible with these reagents. The Human Broad Immune Profiling CyTOF Panel, 20 Antibodies (PN 201339B/C) was developed to be compatible with these commercial WB stabilizers and for use in pharmaceutical and clinical research. Unique advantages of the Human Broad Immune Profiling CyTOF Panel for whole blood immune profiling Unlike other commercially available high-parameter immune profiling products, the Human Broad Immune Profiling CyTOF Panel is optimized for fresh or fixed whole blood and PBMC samples to capture key markers and identify over 30 cell populations of interest (Figure 2A). This product offers unique advantages for longitudinal and multi-site studies with the option to preserve and store samples as needed – enabling more flexibility to stain and process samples at any time and from any location

Methods Antibody panel – the Human Broad Immune Profiling CyTOF Panel was pooled to form an antibody cocktail and frozen (–80 °C) as single-use aliquots for this study. Table 1. The antibody targets and clones in the Human Broad Immune Profiling CyTOF Panel, 20 Antibodies Figure 2. The Human Broad Immune Profiling CyTOF Panel offers workflow flexibility with a multitude of different cell staining and processing workflows. (A) A list of the major and minor cell subsets identified by the Human Broad Immune Profiling CyTOF Panel. (B) The panel is compatible with staining of freshly drawn blood, PAXgene® Blood DNA Tubes for short-term storage, PROT1 and Cytodelics stabilization reagents for long-term storage, freezing and thawing of antibody-stained samples1, and sample multiplexing for the analysis of WB. WB handling, staining, stabilization and downstream processing – WB from three healthy adult donors was collected in BD Vacutainer® EDTA Tubes over the course of three weeks (one donor each week). WB was stored at 4 °C and processed on the day of collection. For details of the protocol performed in this experiment, refer to A 20-Marker Core Immune Flow Cytometry Panel Optimized for Flexible Whole Blood Preservation Workflows Application Note (FLDM-01322)2. Three staining and cell processing workflows were tested: 1) fresh-stain workflow, 2) stain-preserve workflow, 3) preserve-stain workflow (Figure 3). During the processing of each donor with the freshstain workflow, a longitudinal control PROT1 stabilized WB sample was stained in parallel. Acquisition and analysis – Pre-acquisition processing was performed as instructed in the Maxpar® Cell Staining With Fresh Fix User Guide (FLDM-01319)3. All samples were acquired on CyTOF XT™. Debarcoding was performed using CyTOF Software v8.1.0 to obtain individual FCS files. Manual gating was performed using Cytobank Analysis software (cytobank.org).

Results The Human Broad Immune Profiling CyTOF Panel is compatible with whole blood stabilization reagents Frozen CyTOF antibody cocktails are stable Figure 4. Representative dot plots demonstrate the compatibility of the Human Broad Immune Profiling CyTOF Panel with PROT1 and Cytodelics stabilization reagents. These density dot plots are derived from traditional manual gating of CyTOF flow cytometry data using Cytobank Analysis software. Representative plots from a single donor across different workflows and stabilization reagents are shown here. Population names, key markers and parent population are shown to the left of the plots. Figure 5. Metal-conjugated antibodies in a frozen cocktail retain binding activity and yield comparable signal intensities over time. Panel antibodies were combined to make an antibody cocktail and frozen (–80 °C) as singleuse aliquots. The frozen cocktail was thawed one, seven and 14 days after freezing and used to stain aliquots of PROT1 stabilized/frozen WB from a single draw of one donor to compare signal intensities of different markers over time. CyTOF flow cytometry data was analyzed by manual gating and the signal intensities of different markers in key immune cell populations are shown. The average %CV for the median signal intensity was 13.1% and ranged between 4.2–21.9%.

Conclusion CyTOF technology with the Human Broad Immune Profiling CyTOF Panel offers unique advantages for longitudinal and multi-site studies with the option to preserve and store samples as needed – enabling more flexibility to stain and process samples at any time and from any location. Broad immune profiling. The Human Broad Immune Profiling CyTOF Panel identifies 32 cell populations. Flexible experimental workflows. The panel is compatible with PROT1 and Cytodelics whole blood stabilization reagents, which overcomes the logistical challenges of phenotyping WB processed by different collection sites. Compatible with multiplexing. Fresh or stabilized samples can be multiplexed before or after antibody staining to reduce processing times and technical variability4. Freezing antibody cocktails. This unique feature of CyTOF flow cytometry5 ensures batch-to-batch consistency in longitudinal clinical research.

Conflict of Interest No. Standard BioTools.

Title DIAGNOSTIC AND PREDICTIVE USEFULNESS OF BAL LEUKOCYTE PROFILE ANALYZED BY FLOW CYTOMETRY IN ILD AND LUNG INFECTION **Code** 16

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Topic Others

Abstract

Introduction Interstitial lung diseases (ILD) represent a diverse group of disorders that primarily affect the pulmonary interstitium and, less commonly, involve the alveolar and vascular epithelium. Overlapping clinical, radiological and histopathological features make proper classification difficult, requiring multiple complementary methodologies including flow cytometry of bronchoalveolar lavages (BAL). BAL is commonly used in ILD evaluation since it allows sampling the lower respiratory tract. However, the diagnostic and prognostic usefulness of BAL cell counts by flow cytometry in ILD and pulmonary infection has been poorly evaluated.

Methods This retrospective study analyzed BAL flow cytometry data from 1074 real-life patients, quantifying alveolar macrophages, CD4/CD8 lymphocytes, neutrophils, eosinophils, and CD1a+ Langerhans cells and retrospectively correlated with clinical, radiological, anatomopathological, functional/spirometry and evolutionary data.

Results Clustering and logistic regression analyses identified seven distinct leukocyte profiles: 1) Lymphocytic (associated with hypersensitivity pneumonitis, cryptogenic organizing pneumonia, and lymphocytic interstitial pneumonia); 2) Sarcoidosis; 3) Macrophagic (including nonspecific interstitial pneumonia, desquamative interstitial pneumonitis, pneumoconiosis, and unclassifiable ILD); 4) Neutrophilic (including usual interstitial pneumonia, respiratory bronchiolitis ILD, and acute interstitial pneumonia); 5) Infectious diseases; 6) Eosinophilic ILD; and 7) Langerhans cell histiocytosis. The estimated leukocyte profiles were associated with different overall survival (OS) outcomes. Neutrophilic profiles, both infectious and non-infectious, correlated with poorer OS, particularly in patients without pulmonary fibrosis. Furthermore, corticosteroids and other immunosuppressive therapies did not show significant OS differences across leukocyte profiles. Cut-offs with predictive value were established at 7% and 5% for lymphocytes and neutrophils, respectively. Three risk stratification groups (Risk-LN) were established: FAVORABLE (lymphocytes >7% and neutrophils <5%), INTERMEDIATE (rest of patients) and UNFAVORABLE (lymphocytes <7% and neutrophils >5%), showing 75-percentile overall survival (OS) of 10.0±1.4, 5.8±0.6 and 3.0±0.3 years ($p<0.001$), respectively. A scoring model combining Risk-LN and the age of the patients, with great predictive capacity for OS on fibrotic and non-fibrotic ILDs is proposed. This score is an independent predictive factor (HR=1.859, P=0.002), complementary to the fibrosis status (HR=2.081, P<0.001) and the type of treatment. The logistic regression formulas are accessible to citometrists around the world at "https://bal-ildcalculator.imib.es/", where they will be able to enter BAL leukocyte subset counts of individual patients to estimate the pathology most likely associated with this cellular profile.

Conclusion These results support BAL flow cytometry as a rapid, accurate and reliable tool to aid in the classification of interstitial lung diseases based on immune cell profiles, providing valuable predictive information and contributing to the clinical management of fibrotic and non-fibrotic ILDs from the time of diagnosis and to personalized therapeutic approaches.

Conflict of Interest No.

Title MASS AND COMPUTATIONAL CYTOMETRY IDENTIFY CIRCULATING ANTIGEN-PRESENTING CELLS DURING ACUTE SARS-COV-2 INFECTION AS PREDICTORS OF SUBSEQUENT DEVELOPMENT OF LONG-COVID **Code** 17

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Topic Immunology

Abstract

Introduction Long COVID (LC) has become a significant public health issue, affecting an estimated 10%-30% of individuals infected with SARS-CoV-2—over 60 million people worldwide. It is defined as a chronic condition persisting for at least two months following SARS-CoV-2 infection, characterized by a wide range of symptoms or conditions unexplained by alternative diagnoses. Commonly reported symptoms include fatigue, brain fog, and exercise intolerance. Several hypotheses implicate abnormal immune responses, but the underlying pathophysiology remains unclear. Indeed, it is currently unknown whether LC patients could have a differential immune profile that might have predisposed them to subsequently develop such condition. Hence, we hereby aimed to address such issue by performing a comprehensive characterization of the circulating immunome from newly diagnosed COVID-19 patients during the first wave to determine whether they carried a differential immune fingerprint that might have predisposed them to develop such condition.

Methods We conducted a retrospective analysis of patients from whom peripheral blood mononuclear cells (PBMC) had been obtained at the time of hospitalization due to PCR-confirmed COVID-19 diagnosis during the first wave of the pandemic (March-April 2020), as well as 3 months after hospital discharge. A total of 10 patients who subsequently developed LC in the following 5 years were identified, as well as 13 non-LC patients who did not (matched by age, sex, and disease severity during the acute phase of the infection). PBMC immune profiling was applied using a 40-plex mass cytometry panel, combining both classical hierarchical gating strategies and unsupervised analyses. Dimensionality reduction and clustering algorithms were applied to enable an unbiased approach.

Results A total of 48 different immune cell subsets were identified following hierarchical gating approaches. Nevertheless, none of them was differentially expressed between LC patients referred to those who did not, neither at diagnosis nor 3 months after hospital discharge. However, an unsupervised clustering approach identified a total 70 immune cell clusters, 11 of which displayed significant temporal and cohort-dependent variations. Further validation revealed that CCR5 expression on conventional dendritic cells (cDCs) was increased at the time of hospital admission in patients who subsequently developed LC, returning to normal levels 3 months after hospital discharge. On the contrary, CD49d expression of intermediate monocytes was increased on patients who did not subsequently develop LC, with their levels coming back to normal over time. Last, but not least, CD56 expression on cDC was constitutively increased in LC patients not just during the acute phase of the disease, but also after hospital discharge.

Conclusion Our findings suggest that individuals who developed LC exhibited dysregulations in subsets of antigen-presenting cells (APCs) during acute SARS-CoV-2 infection, with some of these alterations not coming back to normal following resolution of the infection. These alterations may therefore contribute to long-term immune dysfunction, potentially shaping the pathogenesis of LC by affecting both innate and adaptive immune responses.

Conflict of Interest No.

Title THE CLINICAL SIGNIFICANCE OF PHENOTYPIC BONE MARROW MONOCYTE FEATURES IN MYELODYSPLASTIC NEOPLASMS **Code** 18

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Topic Hematology

Abstract

Introduction Recently, the boundaries between myelodysplastic neoplasms (MDS) and chronic myelomonocytic leukemia (CMML) have been reviewed as several works have shown that 4.8% - 20% of patients with MDS may progress to CMML, depending on the criteria used to define CMML. In a previous study analyzing the immunophenotypic features of bone marrow (BM) in patients with MDS at diagnosis, 27% showed an increased percentage of monocytes compared to normal BM. This feature, together with increase of CD34+ myeloid progenitors and decrease of B-cell progenitors were associated with a poor outcome of the patients. We reexamined the evolution of these patients in order to see their outcome and if it was possible to predict progression to CMML.

Methods Patients were diagnosed between 2005 and 2012, using peripheral blood counts and BM cytology, cytogenetics and immunophenotyping with a 4-color flow cytometric protocol. Analysis was made in the Infinicyt 1.7 software. The cases were classified according to the WHO 2016 criteria. Diagnosis of CMML was made when peripheral blood monocytes were stable ≥1x109/l.

Results Among 95 cases, we found 26 that had total monocytes / total nucleated cells above the normal range (>5.5%). Among them, 6 developed full-blown CMML during their evolution (2-28 months). Patients progressing to CMML had, at diagnosis, a higher percentage of total monocytes / TNC (median 10.1% x 7.8%), a higher percentage of CD16+ monocytes / TNCs (median 2.4% x 0.96). The percentage of CD16+ cells among total monocytes was very variable and there was no statistical difference between both groups. BM B-cell progenitors were absent at diagnosis in all cases developing CMML. Age and percentage of BM CD34+ myeloid cells were not different in both groups

Conclusion increase in BM monocytic lineage is frequent in MDS, although no monocytosis is detected in peripheral blood. However, it may be indicative of a progression to CMML.

Conflict of Interest No.

Title ALLOWABLE TOTAL ERROR IN CD34 CELL ANALYSIS BY FLOW CYTOMETRY BASED ON STATE OF THE ART USING SPANISH EQAS DATA **Code** 19

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Topic Accreditation and management

Abstract

Introduction CD34+ hematopoietic stem cell (HSC) enumeration is performed by flow cytometry to guide clinical decisions. Variability in enumeration arises from biological factors, assay components, and technology. External quality assurance schemes (EQAS) train participants to minimize inter-laboratory variations. The goal is to estimate total error (TE) values for CD34 cell enumeration using state-of-the-art (SOTA) methods with EQA data and to define quality specifications by comparing TE using different cutoffs.

Methods A total of 3,994 results from 40 laboratories were collected over 11 years (2011-2022) as part of the IC-2 Stem Cells Scheme of the GECLID Program that includes absolute numbers of CD34 cells. The data were analyzed in two periods: 2011-2016 and 2017-2022. The TE value achieved by at least 60 %, 70 %, 80 %, and 90 % of laboratories was calculated across the two different periods and at various levels of CD34 cell counts: above 25, 25 to 15, and under 15 cells/µL.

Results A decrease in the SOTA-based TE for CD34 cell enumeration was observed in the most recent period in 2017-2021 compared with 2012-2016. A significant increase of P75 TE values in the low CD34 range (<15 cells/µL) levels was found ($p<0.001$).

Conclusion Technical advancements contribute to the decrease TE over time. The TE of CD34 cell FC counts is measure-dependent, making it responsive to precision enhancement strategies. The TE measured by EQAS in this study may serve as a quality specification for implementing ISO 15189 standards in clinical laboratories for CD34 cell enumeration.

Conflict of Interest No.

Title MONITORING MYELOID PATTERNS OF MATURATION DURING ACUTE MYELOBLASTIC LEUKEMIA: MIND THE ABNORMAL! **Code** 21

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Topic Hematology

Abstract

Introduction Normal bone marrow (BM) myeloid maturation shows highly reproducible patterns of antigen expression. Chemotherapy for acute myeloblastic leukaemia (AML) causes initial myelosuppression, and then BM cells are restored.This study describes the pattern of neutrophil and monocyte maturation throughout AML treatment and investigates whether this information is clinically relevant.

Methods Between 2019 and March 2025, 27 out of 56 AML patients seen at our hospital entered this study. Inclusion criteria were newly diagnosed de novo AML patients (median age of 54 years, range 15-75) candidates for intensive chemotherapy. The series included four acute promyelocytic leukemia (APL) and nine adverse-risk cytogenetics AML. BM samples (n=124) were analyzed by multiparametric flow cytometry immunophenotyping (MFCI) at different time points during therapy. Twenty samples were excluded (6 relapses and 14 under hypomethylating agent therapy). The remaining 104 studies, performed after induction (n=27), during consolidation/maintenance (C/M) (n=23), and after completion of treatment (n=54) were evaluated. MFCI included measurable residual disease (MRD) assessment and examination of neutrophil and monocyte maturation patterns using the EuroFlow (EF) AML/MDS antibody panel (Tubes 1-2). Samples were prepared according to EF standard operating procedure. Infinicyt was used for manual data analysis. The maturation patterns were classified as normal (for both lineages), partial (one lineage deviations), and abnormal (deviations in both). MFCI findings were correlated with hematological response, MRD data and clinical outcome. For this last purpose, 41 samples from 11 non-APL patients who did not receive frontline allogeneic stem-cell transplantation (allo-SCT) were analysed.

Results Maturation patterns changed during therapy phases ($p<0.0001$). Normal patterns increased as therapy progressed: 4/27 (14.8%) after induction, 5/23 (21.7%) in C/M, and 26/54 (48.1%) at end of therapy. Conversely, abnormal patterns were more frequently observed after induction (17/27; 63%) than C/M (3/23; 13%), and end of therapy (7/54; 12.9%). Normal vs. non-normal patterns increased significantly after 6 months from the end of treatment ($p=0.02$), and all abnormal patterns were described within the first 8 months. None of the maturation patterns were associated with a specific hematological response. Complete remission was most frequently associated with an abnormal pattern after induction (12/19; 63.2%), a partial pattern in C/M (12/15; 80%) and a normal pattern at end of therapy (26/50; 52%). The frequency of normal/partial/abnormal patterns was 13%/34.8%/52.2% in MRD positive (n=23) studies, and 39.5%/42%/18.5% in MRD negative (n=81) studies ($p=0.0027$). After induction, each maturation pattern showed about 50% of cases with positive MRD. Finally, correlation of patterns and clinical outcome revealed that 8/11 patients who did not receive allo-SCT presented normal or partial patterns at the end of therapy: six patients remained in complete remission after a median follow-up of 42 months (range 19-81), and two relapsed. In contrast, abnormal patterns at the end of therapy always preceded relapse. Two patients showed abnormal patterns in all their determinations. One of them, diagnosed with NPM1mut AML, achieved complete molecular remission but at relapse, exhibited a different NPM1 mutation with a variant allele frequency of 8.7%. In the last patient, the shift from a partial to an abnormal pattern coincided with MRD positivity and preceded relapse one month later.

Conclusion The maturation pattern changes during therapy and should normalize as therapy progresses. In this series, a normal pattern at the end of therapy did not preclude relapse, but all abnormal patterns preceded relapse. Their identification might be a useful adjunct to classical MRD as an early predictor of relapse in patients not undergoing allo-SCT. Larger studies are warranted to confirm these findings.

Conflict of Interest No.

Title PRIMARY PLEURAL EFFUSION LYMPHOMA: A CHALLENGING DIAGNOSIS INVOLVING FLOW CYTOMETRY **Code** 22

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Topic Hematology

Abstract

Introduction Serous effusions are observed in several malignancies, with lymphoma being a common cause. T-cell lymphomas are more frequently associated with serous effusions, whereas they are rare in B-cell lymphomas. Primary effusion lymphoma is a rare and aggressive B-cell lymphoma characterized by the presence of tumor cells in body cavities such as the pleura, pericardium or peritoneum, without a visible solid tumor. According to the World Health Organization (WHO), effusion lymphoma is frequently associated with human herpesvirus type 8 (HHV-8) infection and is commonly seen in immunocompromised individuals. Its incidence is highest in individuals between the ages of 50 and 70 years. Clinically, patients usually present with fever, weight loss, fatigue and dyspnea as well as pleural effusions. Diagnosis of this lymphoma can be challenging due to its nonspecific clinical presentation and flow cytometry plays a crucial role in it.

Methods A 79-year-old male patient was admitted to the emergency department for arrhythmia and progressive dyspnea of one month's evolution. He also presented edema in lower limbs and increased abdominal perimeter. He did not report chest pain, fever, cough or expectoration. He has a personal history of hypertension, type 2 diabetes, occasional cigar smoking, osteoarthritis and diabetic retinopathy. A year earlier he had had a massive pleural effusion in which no malignancy was detected.
Blood count data were normal except for a decrease in lymphocytes [0.4 10^3/µL (1.1–4.0); 5.4% (20.0–44.0)]. The coagulation study showed alterations with increased prothrombin time, reduced prothrombin activity, elevated INR and prolonged PTT. Blood gases analysis showed low oxygen saturation (24.5%), high lactate levels [2.20 mmol/L (< 1.80 mmol/L)] and pCO2 [55 mmHg (38–48)]. High levels of LDH [402 U/L (120–246 U/L)] and CRP [54.3 mg/L (< 5.0 mg/L)] were also observed. Urinalysis showed no alterations.

Results The immunophenotypical report of pleural fluid revealed the presence of 93.80% of big cells, with a low positive expression for CD45, CD4 and CD123. A high positive expression in CD38, citoCD30, CD43, CD44, CD71 and CD45RA was observed. There was no expression of B-cell markers (CD19, CD20, CD22, citoCD79b, citoCD79a, citoIgM, BCL-2), T-cell markers (CD3, citoCD3, CD8, CD2, CD5, CD7, TCL1, CD57), NK markers (CD56, CD16); precursor cells markers (CD34, CD117, Tdt) and other markers such as CD33, CD36, CD200, CD81, HLA-DR, CD11b and CD27. Given this immunophenotypic profile and the patient's clinical presentation, we wanted to confirm the diagnosis of effusion lymphoma so both pleural fluid and peripheral blood specimens were tested for HHV-8 detection via PCR. Both samples were found to be positive for the virus.

Conclusion The diagnosis of primary effusion lymphoma presents a significant challenge, particularly in the context of flow cytometry. It often requires the use of multiple cellular markers before a well-supported clinical suspicion can be established. This challenge is further exacerbated by the fact that not all efussion lymphoma cases are HHV-8 positive. Furthermore, the clinical presentation of this entity can be nonspecific, often mimicking other conditions such as infections or autoimmune diseases, which complicates the diagnostic process. Imaging studies, such as chest X-rays or CT scans, may reveal fluid accumulation in body cavities, but these findings are not exclusive to effusion lymphoma and require further confirmation through cytological and molecular analysis. Consequently, a multidimensional approach, combining clinical evaluation, histopathological examination, flow cytometry, HHV-8 PCR, and other biomarkers, is essential for the accurate and comprehensive diagnosis of this rare and aggressive lymphoma.

Conflict of Interest No. The author declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Title NANOPLASTIC CONTAMINATION ACROSS COMMON BEVERAGES AND INFANT FOOD: AN ASSESSMENT OF PACKAGING INFLUENCE **Code** 24

Authors Names Roser Salvia(1); Carlos Soriano(1); Irene Casanovas(1); Michael D. Ward(2); Jordi Petriz(1)

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Topic Biotechnology and new applications

Abstract

Introduction Nanoplastics (NPs) are a significant environmental pollutant increasingly recognized as a global health threat. NP bioaccumulation has been documented in human tissues, including blood and placenta, and in vivo studies have shown its adverse effects on the digestive, respiratory, reproductive, neurological, and cardiovascular systems. Considering ingestion as a significant entry pathway for NPs into the human organism, this research explores NP contamination in commercial waters, soft drinks, and infant formula, products with widespread consumption.

Methods Thirteen samples, including commercial waters (n=8), infant follow-on formula (n=1), lyophilized cereal porridge (n=1), and three soft drinks in aluminum, plastic, and crystal containers, were analyzed for NP content. Sixteen samples of pyrogen-free water were included as negative controls. Lyophilized follow-on formula and cereal porridge samples underwent organic matter digestion using 1% potassium hydroxide (KOH) at 60°C in a dry block for 10 days. A 20 µl aliquot of each digested sample and soft drink was diluted in 1 ml pyrogen-free water and subsequently stained with 2 µl of Nile Red, achieving a final concentration of 0.2 µg/ml. The staining process was conducted for 15 minutes at room temperature in the absence of light. Commercial waters and pyrogen-free water (1 ml) were stained with Nile Red. NP accumulation was assessed in triplicate. Submicron microspheres were used for flow cytometry calibration, and all samples were analyzed using the Attune™ NxT flow cytometer. Sample preparation was conducted under controlled conditions within a Biosafety Level 2+ (BSL2+) environment.

Results Pyrogen-free water was analyzed to establish the negative control for NP concentration, yielding 5.03 ± 2.06 events/µl. Compared to pyrogen-free water, infant formula and cereal porridge showed very low NP concentrations, with values of 10.27 ± 6.85 and 6.78 ± 2.27 events/µl, respectively, following triplicate analyses of six samples. NPs were detected at significantly elevated concentrations ($p < 0.0001$) in 5 of the 8 plastic-bottled commercial water samples analyzed in triplicate, with a range of 5.93 to 124.9 events/µl.

Conclusion NPs, as ubiquitous contaminants, can be ingested by organisms through food and drink. Potential nanoplastic (NP) contamination in commercial water can arise from factors such as source water contamination, filtration, and packaging. The presence of very low NP concentrations in infant food suggests effective stringent quality control. Finally, NP presence in soft drinks was not affected by container type. While soft drink beverages have higher NP levels than water, container type had no effect on NP presence in soft drinks.

Conflict of Interest Yes. (2) Research Scientist, Thermo Fisher Scientific Inc.

Title IMAGING FLOW CYTOMETRY FOR ACCURATE SINGLET IDENTIFICATION **Code** 25

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Topic Biotechnology and new applications

Abstract

Introduction Flow cytometry is a widely used and precise method for single-cell analysis. Accurate characterization of a sample relies on distinguishing true single cells from non-single-cell events, such as doublets or aggregates, acquired by the flow cytometer. This distinction is typically achieved by evaluating the forward light scatter pulse's area or width versus its height. However, the distributions of these parameters for single cells, doublets, and other events often overlap. This overlap raises concerns that standard gating methods may inadvertently exclude true single cells while failing to remove doublets that fall within the "singlet gate". In this study, we used imaging flow cytometry to better characterize events conventionally gated as doublets.

Methods Bone marrow and peripheral blood samples, the EGFP-K562 cell line, and fluorescent microbeads (Thermo Fisher and Sysmex) were analyzed. Samples were acquired using the Invitrogen™ Attune™ CytPix™ flow cytometer (Thermo Fisher) without washing steps. Automated image analysis translated event features into morphometric parameters, which were combined with standard fluorescence and scatter parameters. A standard "doublet gate" was established based on forward light scatter, side light scatter and fluorescence (DNA content). Circularity versus major diameter plots were used to further characterize events within the doublet region.

Results Three distinct populations were identified within the "doublet gate" based on circularity and major diameter: (1) events with high circularity and low diameter, which imaging flow cytometry confirmed were single cells (mean percentage ± SD in 10 bone marrow samples = 37.44% ± 19.59% and can be found up to 50%); (2) events with intermediate circularity and high diameter (doublets); and (3) events with low circularity and high diameter (coincident events). These findings were independent of the gating method used (light scatter or fluorescence). Microbeads were less likely to appear in the doublet gate and, when they did, less likely to be singlets. In contrast, when spiking K562 in peripheral blood samples, the single-cell K562 blasts were more likely to appear in the doublet gate, indicating that large cells are more likely to fall within the doublet compartment.

Conclusion Accurate identification of singlets and removal of false-positive results are crucial for both flow cytometry analysis and cell sorting. The doublet compartment is heterogeneous, and standard doublet discrimination strategies can exclude single cells. Pathological, larger, activated, or proliferating cells—those often of greatest interest—are more likely to be misclassified as doublets, potentially leading to the underestimation or exclusion of key cell populations. Care must be put into the routine doublet gating step, and we propose defining the singlet gate in the last steps of the analysis when working with larger or activated cells of interest.

Conflict of Interest Yes. (2) Research Scientist, Thermo Fisher Scientific, Inc.

Title CHARACTERIZATION OF ALKALINE PHOSPHATE ACTIVITY IN HEMATOPOIETIC STEM CELLS AS A PREDICTOR OF TRANSPLANT EFFICIENCY IN LEUKAPHERESIS PRODUCTS **Code** 26

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Topic Hematology

Abstract

Introduction Accurate prediction of long-term engraftment following hematopoietic stem cell transplantation is crucial for optimizing clinical outcomes. While routinely used, CD34+ cell counts often fail to fully capture engraftment potential, especially in poor mobilizers. We investigate the clinical utility of a novel flow cytometric method, which integrates CD34+ cell counts and alkaline phosphatase (ALP) activity measurements, to predict long-term engraftment. We propose that APLS+CD34+CD45lo cells have the potential to predict long-term engraftment.

Methods Leukapheresis products and mobilized blood samples were collected for this study from 21 multiple myeloma (MM) patients and 21 healthy donors (HD) undergoing mobilization treatment at the Blood Bank (Germans Trias i Pujol Hospital), in accordance with ethical approval and informed consent. Following a minimal sample perturbation protocol, all samples were incubated with Hoechst 33342, CD34-APC, CD45-PE-Cy7, and APLS (Alkaline Phosphatase Live Stain) for 20 minutes at room temperature in the dark. Samples were acquired using the Attune NxT flow cytometer (Thermo Fisher).

Results Compared to HD, MM patients, after bone marrow ablation, exhibited significantly lower CD34+ cell mobilization in peripheral blood (0.135 ± 0.082 vs. 0.261 ± 0.133) and leukapheresis products (0.681 ± 0.479 vs. 1.020 ± 0.480). Similarly, APLShighCD34+ progenitor cell counts in leukapheresis products were significantly reduced in MM patients (28.16 ± 18.96) relative to HD (46.14 ± 33.39). Poor mobilizers, patients exhibiting low CD34+ cell counts, demonstrate a notable absence of APLS+ expression within the CD34+ cell compartment.

Conclusion The observed reduction in APLShighCD34+ progenitor cell counts in these patients strongly suggests a compromised engraftment potential. This finding highlights the need for more comprehensive engraftment prediction tools. We suggest that ALP activity, reflecting engraftment capacity, may have significant prognostic value in predicting transplant outcomes. This would allow for risk stratification and individualized post-transplant interventions, along with the refinement of stem cell isolation techniques to maximize engraftment success.

Conflict of Interest Yes. (2) Research Scientist, Thermo Fisher Scientific, Inc.

Title NEW FLOW CYTOMETRY PROTOCOL FOR HIGH-SENSITIVITY DETECTION OF MEASURABLE RESIDUAL DISEASE IN HEMATOLOGIC MALIGNANCIES IN CLINICAL LABORATORIES **Code** 27

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Topic Hematology

Abstract

Introduction Efficient bulk lysis protocols are essential for accurate MRD assessment in leukemia by flow cytometry. Higher cell acquisition enhances detection by increasing the likelihood of identifying rare leukemic populations, while insufficient cell numbers elevate the risk of false negatives. However, extended staining and acquisition times, along with high service demand, hinder routine MRD studies in many hospital laboratories, often requiring outsourcing. This study introduces FACSLyse-Bulk, a streamlined protocol that enables clinical flow cytometry labs with standard resources and limited personnel to perform MRD analyses efficiently with reduced processing time.

Methods A total of 271 human samples were analyzed, comprising 226 bone marrow and 45 peripheral blood samples, intended for MRD assessment and the detection of rare populations, such as those in mastocytosis. All samples were processed using the FACSLyse-Bulk protocol and analyzed within 24 hours of collection as briefly summarized below: Stain a minimum sample volume containing 8 million WBC. Cells were washed twice with wash buffer (+ 3% BSA), centrifuged at 1800 rpm for 5 min., and the supernatant was removed. Surface antigens were labeled using fluorescence-conjugated antibodies for 15 min. at RT in the dark, following the manufacturer's recommended antibody concentration for 1 million cells/test. The cells were then washed, centrifuged, and the supernatant was removed. After gentle vortexing, 2 mL of FACS lysing solution was added. Following a 6-minute incubation at RT, the suspension was centrifuged, 5 mL of wash buffer was added, and the cells were centrifuged again before supernatant removal. For intracellular staining, cells were incubated with appropriate antibodies for 15 minutes at RT, washed, and resuspended in 2 mL of wash buffer. Samples were analyzed in a BD FACSCanto II™ flow cytometer and data were analyzed with Infinicyt™ software. To ensure comparable results, PMT voltages were optimized to enhance the resolution of dim cell populations using unlabeled lysed whole blood cells. The obtained mean fluorescence intensity (MFI) target values were subsequently used to maintain instrument standardization during calibrations.

Results Although the lysing solution contains formaldehyde, no evidence of tandem dye degradation was observed in the studied markers. Membrane fixation by the lysing solution prevents capping and receptor-mediated endocytosis, while also allowing sample acquisition to be postponed until the cytometer becomes available. The protocol minimizes debris and doublet formation and does not significantly affect cell complexity or labeling resolution. For the bone marrow and peripheral blood samples tested, the mean LOD values were 0.0003978% and 0.0009946%, respectively, while the mean LOQ values were 0.0005957% and 0.0014891%. Most bone marrow aspirates had a volume of less than 2 mL, which did not pose a limitation, as the mean sample volume required to stain 8 million cells was 695.38 µL. In contrast, peripheral blood samples required a larger volume of 1347.22 µL, as cell counts are typically lower in blood than in bone marrow.

Conclusion The FACSLyse-Bulk protocol is a rapid and efficient method (<90 min) that requires minimal sample volume while achieving low limits of detection (LOD) and quantification (LOQ) (<0.001), with 99.26% of samples meeting these criteria. The protocol uses 5 mL FACS tubes throughout sample processing which simplifies and speeds up the process and reduces potential sample handling errors. Therefore, key advantages of the FACSLyse-Bulk protocol include minimal sample requirements and reduced processing times, enhancing workflow efficiency and enabling routine implementation in clinical flow cytometry laboratories.

Conflict of Interest No.

Title AN INTERDISCIPLINARY APPROACH TO SUBMICRON SORTING IN A SHARED RESOURCE LABORATORY (SRL) SETTING **Code** 29

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Topic Biotechnology and new applications

Abstract

Introduction In the post-COVID era, Shared Resource Laboratories (SRLs) are at the forefront of increased demand for sorting beyond traditional immunological applications. Research directions are moving toward sorting submicron biological samples (less than 2µm), particularly microbes, organelles, and extracellular vesicles. SRLs integrate bench research, biotechnology, and customer service within a changing global research landscape that imposes economic pressures on fee-for-service facilities. Fewer SRLs regularly perform submicron sorting; it is constrained by the limitations of existing instrumentation, time required, and knowledge base for sorting non-traditional samples. This is further complicated by challenges in accommodating microbial sorting, stemming from misconceptions around sample pathogenicity and instrument contamination, and the absence of formalized best practices for maintenance. An interdisciplinary approach has enabled successful execution of diverse submicron sorting for novel applications such as animal-microbe symbioses, synthetic biology, and microbiome analysis for single-cell or particle genomics, alongside traditional sorting, in a general SRL setting.

Methods Key disciplines considered were operations management, microbiology, instrumentation/physics, and imaging. Instruments were tested to determine limit of detection, sensitivity, and sort accuracy. Operations management involved strategic scheduling, instrument preparation, and decontamination to optimize instrument and staff availability. Consideration of factors beyond cell number and panel design was required to select a sorter and schedule sufficient time for experiments. Pilot experiments supported optimization of future sort scheduling. Open-ended scheduling was appropriate for sorts requiring low flow rates or unknown quantities. Cleaning agents were carefully selected. Sort performance and gating strategies were validated by microscopy, cultivation, and sequencing when relevant. Open communication and feedback from SRL researchers was key in validating procedures.

Results From January 2022 to January 2025, an SRL staff researcher responsible for operating instrumentation for submicron experiments performed a total of 267 sorts, 80 of which were submicron (29.96%), plus an additional 42 non-traditional application sorts (15.73%) across three sorters, accompanied by the researchers conducting the experiments. 555 billable instrument hours were tracked over 55 submicron sorts performed on a BD FACSAria Fusion sorter shared with multiple SRL researchers and trained researchers for facility sorting. An estimated 250 hours were logged across two other sorters over 25 additional submicron sorts. Additional non-billable hours were dedicated to literature review, service oversight, testing, continuing education, and protocol documentation. Extensive inspections and maintenance were regularly conducted. There were 0 incidences of sample-related sorter contamination. New instrumentation-related protocols were created, and existing protocols were documented.

Conclusion The interdisciplinary approach to submicron sorting resulted in cutting edge research spanning multiple fields, paving the way for mainstreaming emerging and novel applications. Initial investment in non-billable hours was high in the short-term time frame examined. However, these hours do not need to be repeated as they have generated documentation to transfer the knowledge base to new SRL staff to perform these sorts reliably long-term. Despite economic pressures, this short-term investment advances scientific capabilities in SRLs. Validated protocols enhanced instrument reliability and broadened SRL capabilities, benefiting all users.

Conflict of Interest No. The authors have no potential conflicts of interest to report.

Title PEOPLE LIVING WITH HIV ON ART: IMMUNOLOGICAL EVOLUTION AND ITS RELATIONSHIP WITH AIDS-DEFINING AND NON-DEFINING DISEASES **Code** 31

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Topic Immunology

Abstract

Introduction With the implementation of antiretroviral therapy (ART), mortality associated with Acquired Immunodeficiency Syndrome (AIDS) has significantly decreased. However, non-AIDS-defining illnesses (NADD) have emerged as relevant causes of morbidity and mortality in people living with HIV (PLWH). These conditions may be related to a state of chronic inflammation and persistent immune activation. HIV infection compromises both the quantity and functionality of immune cells, not only increasing susceptibility to severe infections but also the risk of developing B-cell lymphomas, which are frequently associated with Epstein-Barr virus (EBV) infection. Objective: To analyze leukocyte populations, cytokine profiles, and EBV viral load in PLWH on ART to elucidate their impact on disease progression, NADD, and AIDS-defining illnesses (ADD), with special attention to patients with lymphoma.

Methods A total of 265 peripheral blood samples from PLWH on ART at the Hospital Universitario San Ignacio were analyzed, classified according to their clinical stage (E1 65, E2 100, and E3 92), including 8 newly diagnosed lymphoma cases (5 non-Hodgkin lymphomas and 3 Hodgkin lymphomas) and 20 healthy controls. Using next-generation flow cytometry, 52 leukocyte populations were evaluated; EBV viral load was determined by qPCR, and the profile of 27 cytokines was analyzed using Milliplex Luminex technology. Statistical analysis included group comparisons using ANOVA or Kruskal-Wallis for continuous variables and chi-square or Fisher's exact tests for categorical variables. Multivariate logistic regression models were used to identify predictors of ADD and NADD.

Results Flow cytometry analysis revealed significant alterations in lymphocyte subpopulations across different clinical stages of HIV. Advanced disease (stage 3) was characterized by a significant decrease in the total number of lymphocytes, CD4+ T cells (Th) and CD8+ T cells (Tc) at different maturation stages, along with a lower CD4/CD8 ratio ($p<0.05$). Additionally, a reduction in unswitched memory B cells (LBMSS) ($p<0.05$) and switched memory B cells (LBMCS) to IgA and IgG ($p<0.05$) was observed compared to early stages. A significant increase in IL-10 and IFN-γ ($p<0.001$) was observed, along with a reduction in IL-18 ($p<0.005$). Patients with EBV coinfection (10.5%) showed a significant decrease in MBC, Th cells, and the CD4/CD8 ratio. They also exhibited depletion of naïve Th cells (ThN) and central memory Th cells (ThMC) along with an expansion of effector memory Tc cells (TcEM) ($p<0.05$). Patients with a history of virological failure showed an increase in central memory Tc cells (TcMC) and TcEM ($p<0.05$). In contrast, those with immunological failure exhibited a significant decrease in different maturation stages of Th cells, Tc cells, and LBMSS ($p<0.05$). NADD were associated with a reduction in B-cell subpopulations (B cells), particularly LBMCS IgG+ ($p<0.05$). ADD showed a significant decrease in total T cells and Th cells at different maturation stages, along with B memory cell subpopulations ($p<0.05$). Multivariate analysis identified specific B and T cell subpopulations as significant predictors of clinical progression to ADD and NADD.

Conclusion Our findings highlight the complex interplay between immune disruption and chronic inflammation in PLWH on ART. Alterations in T- and B-cell subpopulations, combined with a cytokine profile, suggest a key role in the progression toward ADD and NADD. Additionally, the association with EBV reinforces the need to monitor coinfections and their impact on disease progression. In this context, advanced immune monitoring combined with a comprehensive clinical management approach could be key to optimizing the early identification of at-risk patients and improving long-term clinical outcomes.

Conflict of Interest No.

Title FLOW CYTOMETRY EVALUATION OF IMMUNE DEREGULATION IN AN ATYPICAL HEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS WITH UNKNOWN TRIGGER **Code** 33

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Topic Immunology,Animal Sciences

Abstract

Introduction Hemophagocytic lymphohistiocytosis (HLH) is a rare and severe hyperinflammatory syndrome characterized by a rapidly progressive clinical course and high mortality. It results from excessive immune activation and hypercytokinemia due to an overactivated yet functionally impaired immune response. HLH typically manifests with fever, hepatosplenomegaly, cytopenias, hyperferritinemia, and hemophagocytosis in hematopoietic organs. Diagnosing HLH is challenging, as no single clinical or laboratory parameter possesses sufficient sensitivity or specificity. The HLH-2024 diagnostic criteria and the HScore can aid in diagnosis. Distinguishing between primary (genetic) and secondary (acquired) HLH is difficult, as both share a similar phenotype and are frequently triggered by infections. Flow cytometry (FC) plays a crucial role in HLH diagnosis by evaluating NK cell cytotoxic function and identifying immune dysregulation. Also, genetic testing should be performed to confirm hereditary forms of HLH. Rapid diagnosis and aggressive treatment are essential for improving patient outcomes.

Methods Fresh peripheral blood samples were prepared according to in-house protocols and evaluated with FC.

Results A 41-year-old Brazilian man, residing in Portugal for the past two years, was admitted to the emergency department with complaints of generalized malaise, diffuse myalgias, anorexia, odynophagia, and difficulty ambulating. He reported chronic diarrheal stools over the past 2 years and denied recent travel or other relevant epidemiological exposures. His medical history included poorly controlled type 2 diabetes mellitus and grade 2 obesity. On physical examination, he exhibited proximal tetraparesis, with muscle strength graded as 3/5 in the thighs and 4/5 in the shoulder girdles. Laboratory tests showed anemia, leukocytosis with neutrophilia and eosinophilia, as well as lymphopenia. Renal impairment was noted, with elevated creatinine, proteinuria, hemoglobinuria, and an estimated glomerular filtration rate of 39 mL/min. Additional findings included markedly elevation of: ferritin, triglycerides, lactate dehydrogenase, erythrocyte sedimentation rate, C-reactive protein, and procalcitonin. D-dimer was markedly elevated, and fibrinogen, initially 6.83 g/L, later dropped to 0.7 g/L. Imaging revealed hepatomegaly, and gastrointestinal endoscopy showed ileocolitis and chronic duodenitis. Microbiological testing was negative for infectious agents; however, histological examination of GI mucosa confirmed invasive gastrointestinal aspergillosis. The patient subsequently developed coagulopathy, with progressive elevation of the international normalized ratio (INR), partial thromboplastin and prothrombin time, ultimately progressing to disseminated intravascular coagulation with ADAMTS13 activity of 29%.

Conclusion Immunological studies showed elevated immunoglobulin levels of: IgG1, IgG4 and IgE. Cellular immunity assessment revealed profound lymphopenia of: CD3+, CD4+, CD8+, CD8+/CD45+RA, CD19+, and NK cells; along with upregulation of the CD8+/CD107a+ cells. Additionally, there was downregulation of CD8+/HLA-DR+ and CD4+/HLA-DR+ T lymphocytes, likely due to immunosuppressive therapy. NK CD107a+ degranulation was not evaluated due to the low count of this cell population. TCD8 cells showed abnormally low function. The patient developed severe pancytopenia, leading to two episodes of septic shock with multiorgan failure. The patient awaits the results of genetic testing. Immunosuppressive therapy was initiated with corticosteroids, along with pathogen-directed treatment for active infections. Overall, the clinical presentation and an HScore of 229 are highly suggestive of HLH, with an idiopathic etiology. HLH is a severe, often fatal disease that requires prompt recognition and treatment. FC is an essential diagnostic tool, and its use should be emphasized in suspected cases. Multidisciplinary management is vital to improving survival rates.

Conflict of Interest No.

Title IMMUNE PROFILING OF CONVENTIONAL AND UNCONVENTIONAL T CELLS IN SOFT TISSUE SARCOMA: INSIGHTS FROM FLOW CYTOMETRY AND AI-BASED CLUSTERING **Code** 35

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Topic Solid Tumors

Abstract

Introduction Soft tissue sarcomas (STS) are rare, aggressive malignancies, with a poor prognosis in advanced stages. Despite advances in treatments, the heterogeneity of STS limits effective patient stratification and clinical management. Immune profiles, particularly in peripheral blood, may provide valuable biomarkers for prognosis, as immune cells such as CD8 T cells and NK cells influence tumor progression. Previous studies from our group identified immune profiles in STS patients (C1: immune high, C2: immune intermediate, C3: immune low) correlating decreasingly with survival outcomes. This study aims to analyze peripheral blood and tumour immune cell populations in STS patients using AI-driven flow cytometry, exploring their association with survival and their potential role in immune evasion.

Methods EDTA-treated fresh peripheral blood samples from STS patients (N=31; C1: N=10; C2: N=9; C3: N=12) and healthy donors (HD: N=26) were analyzed using multiparametric flow cytometry. STS tumor samples (N=9) were analyzed similarly to investigate immune infiltration within the tumor microenvironment. Automatic quality control of flow cytometry data was performed using flowAI plugin for FlowJo analysis. Cells were identified using markers for CD3, CD4, CD8, CD56, TCR-γδ, TCR Vα24-Jα18, and CD161. After quality control, FlowSOM clustering was used to identify immune populations, followed by t-SNE for dimensional reduction. Cluster Explorer was used to assess population distribution across groups. Statistical analysis was performed to compare immune profiles between control and STS groups and correlate with survival.

Results "Seven distinct immune populations were identified in peripheral blood: classical CD8+ T cells, CD4+ T cells, CD8- NK cells, CD8+ NK cells, CD4-CD8- γδ T cells, CD8+ γδ T cells, and CD4-CD8- CD161+ T cells. CD8+ NK cells were decreased in both C1 and C2 groups compared to controls. CD8 T cells were elevated in C1 and C2 patients, correlating with better survival. C3 (immune low) patients had lower levels of CD8 T cells than C1 and C2, but their CD8+ NK cell levels were not as low as in C1 and C2. In tumor samples, NK cells, CD8 T cells, and CD4 T cells were the main populations, but CD8+ NK cells were absent, suggesting their limited tumor infiltration even though they were present in the blood.

Conclusion Our findings demonstrate that immune profiles in peripheral blood correlate with survival outcomes in STS patients. Decreased CD8+ NK cells in C1 and C2 groups and increased CD8 T cells in these groups align with better survival, while C3 patients, who exhibit lower CD8 T cells but higher CD8+ NK cells, have worse survival. Despite lower CD8+ NK cells in peripheral blood, these cells were absent in tumor tissues, indicating potential immune evasion mechanisms. AI-driven flow cytometry analysis provides a powerful tool for exploring immune profiles and their implications in STS. These findings suggest that immune profiling could aid in patient stratification, treatment monitoring, and therapeutic targeting of immune cells in STS. Further validation with larger samples is needed to explore these immune signatures in clinical settings.

Conflict of Interest No.

Title ASSESSING THE ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS AGAINST SKIN MICROORGANISMS USING FLOW CYTOMETRY **Code** 36

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Topic Microbiology

Abstract

Introduction The skin microbiome is a dynamic ecosystem vital for skin health, comprising bacteria like Staphylococcus spp. and Cutibacterium acnes, and fungi such as Candida and Malassezia spp. Dysbiosis, or microbial imbalance, can lead to conditions like acne and dermatitis. Current treatments, including anti-inflammatory drugs and antimicrobials, help manage dysbiosis but have limitations, such as microbiome disruption and antimicrobial resistance. Consequently, interest in natural alternatives, particularly essential oils (EOs), is increasing. Rich in bioactive terpenes and terpenoids, EOs target multiple cellular structures, reducing microbial adaptation and resistance. Flow cytometry, a powerful analytical tool, enables precise assessment of antimicrobial activity by distinguishing live and dead microorganisms using fluorescent staining. This study evaluates the antimicrobial effects of eucalyptus, lavender, and thyme EOs, offering insights into their potential as microbiome-friendly therapeutic agents.

Methods A flow cytometry assay was used to assess the antimicrobial activity of eucalyptus, lavender, and thyme EOs against seven skin-associated microorganisms: methicillin-susceptible Staphylococcus aureus (MSSA), methicillin-resistant S. aureus (MRSA), Staphylococcus epidermidis (S. epidermidis), Cutibacterium acnes (C. acnes), Candida albicans (C. albicans), Candida tropicalis (C. tropicalis), and Malassezia furfur (M. furfur). Firstly, minimum inhibitory (MIC) and bactericidal (MBC) concentrations were determined. Then, to further explore EOs antimicrobial effects, microbial cells were exposed to MIC, ½MIC, and ¼MIC of each oil, followed by staining with propidium iodide (PI) and thiazole orange (TO). Flow cytometry was then used to assess viability, membrane integrity, and cell death. Additionally, fluorescence microscopy was performed on yeasts exposed to eucalyptus oil at ¼MIC, providing complementary validation of the flow cytometry findings.

Results The MIC and MBC values of the tested EOs ranged from 0.06% to 2.5% (v/v), with thyme EO showing the lowest values for all microorganisms, indicating its strongest antimicrobial activity. Among bacteria, S. epidermidis had the highest MIC/MBC values, suggesting greater resistance, while C. acnes had the lowest, indicating higher susceptibility. Among yeasts, C. albicans exhibited the highest values, whereas C. tropicalis and M. furfur were more susceptible. Flow cytometry confirmed these findings, validating thyme EO as the most effective, with the highest percentages of dead and injured cells. It also demonstrated that EOs antimicrobial activity is concentration-dependent. S. epidermidis had the highest percentage of live cells, reinforcing its resistance, while C. acnes had the lowest, confirming its susceptibility. C. albicans was more resistant than C. tropicalis. For M. furfur, fluorescence microscopy clarified an apparent lack of TO staining in flow cytometry, confirming viable but unstained cells.

Conclusion The selected EOs exhibited antimicrobial activity against all tested microorganisms. Thyme EO showed the strongest effect. Flow cytometry confirmed the MIC/MBC results, revealing that thyme EO induced the highest percentages of dead and injured cells at all tested concentrations. Among bacteria, S. epidermidis was the most resistant, while C. albicans was the most resistant yeast. For M. furfur, flow cytometry suggested an absence of live cells due to the lack of TO staining, even in untreated samples. However, fluorescence microscopy confirmed that M. furfur cells remained viable but did not stain green like Candida spp. This discrepancy likely results from the yeast's lipid-rich membrane interfering with TO uptake, highlighting potential staining limitations. This work reinforces flow cytometry as a powerful tool to validate EO's antimicrobial effects and support the potential of thyme, eucalyptus, and lavender EOs as natural alternatives for managing skin dysbiosis while preserving microbiome balance.

Conflict of Interest No.

Title A FLOW CYTOMETRIC COMPARATIVE STUDY OF AZITHROMYCIN TOXICITY IN THE FRESHWATER MICROALGA CHLORELLA VULGARIS **Code** 37

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Topic Microbiology

Abstract

Introduction Emerging contaminants (ECs) are substances frequently detected in aquatic ecosystems. These compounds are potentially harmful to living organisms in these environments, including unicellular organisms such as microalgae. Due to their role as primary producers in aquatic food webs and their fundamental role as carbon fixers, several studies have focused on the toxicity of ECs on different microalgal species because of their ecological relevance in these systems. Most microalgal species are unicellular and rich in natural fluorescent pigments, making them exceptionally well-suited for analysis by flow cytometry. These toxicity bioassays typically use axenic microalgal cultures. However, in natural aquatic environments, microalgae coexist in association with a microbiota consisting mainly of heterotrophic bacteria, called phycosphere. Microalgae and bacteria in the phycosphere constantly interact in a dynamic and complex manner, forming associations ranging from mutualism to parasitism. Therefore, these interactions should be considered in toxicity studies with microalgae, as they could affect the potential toxicity of the tested chemical compounds on these microorganisms and, ultimately, on the rest of the microorganisms associated with them.

Methods This study comparatively examines the toxicity exerted by the antibiotic azithromycin (AZT), an emerging contaminant of special ecotoxicological relevance, on the freshwater unicellular microalga Chlorella vulgaris in axenic cultures and in consortium with bacteria isolated from a natural water sample taken from the Abegondo-Cecebre Reservoir (NW of Spain). For this, the microalga is individually exposed to increasing antibiotic concentrations in both types of culture. To assess the toxicity of AZT, growth inhibition was firstly studied and then a panel of cytomic biomarkers was developed to analysed cell viability using the fluorochrome propidium iodide (PI), activation of programmed cell death using Annexin V conjugated to fluorescein and with PI, changes in cytoplasmic and mitochondrial membrane potential´s using the fluorochromes 1,3-dibutylbarbituric acid trimethine oxanol (DiBAC4(3)) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), respectively, as well as oxidative stress analysis by measuring intracellular reactive oxygen species (ROS) levels using the fluorochrome hydroethidine (HE).

Results C. vulgaris response pattern to azithromycin in both types of cultures is the same in most of the analysed parameters: it causes growth inhibition, increases intracellular levels of ROS, activates apoptosis and increases mitochondrial membrane potential, but always reports a more acute alteration in the presence of bacteria in the cultures. Therefore, the mechanism of action of AZT in C. vulgaris cultures grown in the presence of bacteria includes severe damage to the cytoplasmic membrane since the depolarization of these membranes, as well as the loss of microalgal viability were only observed in these non-axenic bioassays.

Conclusion Globally, these results suggest the existence of mechanisms that increase the AZT damage on C. vulgaris cells in the presence of bacteria. Based on these evidences, the next research will focus on studying the potential role that phycosphere bacteria could be acting in the toxicity of emerging contaminants, such as AZT, on microalgal cells of aquatic ecosystems.

Conflict of Interest No. PID2021-127898OB-100

Title A CASE OF AML WITH MASTOCYTIC DIFFERENTIATION. AN AML SUBTYPE? **Code** 38

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Topic Hematology

Abstract

Introduction A 68 years old woman with a 20 years history of JAK2+ essential thrombocythemia attended her monthly blood analysis. Over the past three weeks, she had been experiencing bone pain in the sternum, costal arch, and pelvis, along with severe asthenia. During the last few days, extensive bruising had appeared on her lower extremities. The patient had no history of urticaria or skin lesions. The blood analysis revealed a hemoglobin level of 10.7 g/dL, 5.82x109 WBC/L, 133x109 platelets/L, an LDH of 1,420 U/L, and a D-dimer of 25,097 ng/mL. A peripheral blood smear showed the presence of 28% medium-to-large blasts with granular cytoplasm and a high nuclear-to-cytoplasmic ratio. Due to the suspicion of acute leukemia, a peripheral blood sample was sent to the flow cytometry department for immunophenotyping.

Methods Flow cytometry immunophenotyping was performed on a peripheral blood sample following the EuroFlow ALOT protocol. A subsequent severely hemodiluted bone marrow sample (aspiration was unsuccessful due to a dry tap) was analyzed using the EuroFlow AML panels and an in-house mastocyte panel. The following markers were studied: CD2, cyCD3, CD3, CD4, CD5, CD7, CD9, CD10, CD11b, CD13, CD14, CD15, CD16, CD19, CD22, CD25, CD30, CD33, CD34, CD35, CD36, CD38, CD41a, CD42a, CD42b, CD45, CD56, CD61, CD64, CD71, cyCD79a, CD105, CD117, CD123, CD203c, CD300e, CD304, HLA-DR, cyMPO, NG2, nTdT. The analysis was performed on a 10-color FACSLyric cytometer, calibrated daily with CS&T beads. Data acquisition was carried out using FACSuite cytometer software with EuroFlow tube settings. Analysis was conducted with Infinicyt software.

Results The ALOT study on peripheral blood revealed the presence of 22% blasts CD34+CD7het, among total nucleated cells, negative for cyMPO, CD19 or cyCD3. Immunophenotyping analysis of the hemodiluted bone marrow sample identified 14.65% myeloid blasts (CD34+CD117+CD13++CD33+CD38+/-), among total nucleated cells, with expression of CD56 and a heterogeneous expression of CD25 (+: 20%). Additionally, a small subset of 3.30% of myeloid blasts among total nucleated cells displayed an immunophenotype (CD34dim, CD117++, CD203c+, CD33++, CD11b-, HLA-DR-) and a maturation pattern suggestive of differentiation towards a mastocyte lineage. These blasts also displayed a heterogeneous expression of CD25 (+: 25%). No mature mastocytes were detected in the sample. An NGS study specific for acute leukemia was performed on the sample revealing a pathogenic TP53 mutation, with no evidence of KIT mutations. Cytogenetic analysis identified a complex karyotype. After decline of patient's condition, no further studies were conducted at the request of the family. The patient passed away 22 days after initial analysis.

Conclusion It is well known that myeloid blasts in AML can differentiate into various lineages, such as monocytic, erythroid, or megakaryoblastic (Arber et al., 2016). However, the differentiation of myeloid blasts in AML towards mastocyte lineage remains an uncommon finding in scientific literature. While some authors propose this finding as a precursor stage to myelomastocytic leukemia (Panda et al., 2020), others suggest that AML with mastocytic differentiation (AML-MC) represents a distinct subtype of aggressive AML. This subtype would be characterized by the presence of interstitial mast cells, multilineage dysplasia, complex karyotype, TP53 alterations, and poor prognosis (Do Hwan Kim, 2024). Myeloblasts inmunophenotype in AML-MC has also been described as CD34+CD117+HLA-DR+ with unusually frequent expression of CD56 (57%, all partial) and CD25 (63%, mostly partial)(Xu et al, 2025). Although it was not possible to complete the patient's study, the presence of myeloid blast differentiating towards mastocyte lineage while also sharing previously described characteristics associated with AML-MC, support the recognition of this AML subtype and highlight the need to consider this variant within current prognostic systems.

Conflict of Interest No.

Title ENDOTHELIAL PROGENITOR CELLS AND HYPERTENSIVE DISORDERS IN PREGNANCY: A PRELIMINARY FLOW CYTOMETRY ANALYSIS **Code** 41

Authors Names Alejandra Comins-Boo (1); Juan José Fernández Cabero (1); Juan Irure Ventura (1), Marcos López-Hoyos (1); Laura Diaz López (2); Gema Fernández Fresnedo (3); Lara Belmar Vega (3); David San Segundo Arribas (1)

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Topic Immunology

Abstract

Introduction Preeclampsia is a leading cause of maternal and perinatal morbidity and mortality, characterized by endothelial dysfunction and impaired vascular remodeling. Endothelial progenitor cells (EPCs) and circulating endothelial cells (CECs) are biomarkers of vascular health, potentially reflecting endothelial repair or damage. Angiogenic imbalance, notably increased levels of soluble fms-like tyrosine kinase-1 (sFlt-1) and reduced placental growth factor (PlGF), resulting in an elevated sFlt-1/PlGF ratio, also plays a key role in preeclampsia pathogenesis. This study aimed to evaluate the association between EPCs/CECs and hypertensive disorders in pregnancy, as well as their correlation with angiogenic markers.

Methods Peripheral EDTA blood samples were collected from 38 pregnant women during each trimester. Mononuclear cells were isolated by Ficoll and stained for CD45, CD34, CD31, CD117, CD309 (EPCs), CD146 (CECs), and 7AAD. Flow cytometry was performed on a DxFlex cytometer, acquiring ≥1 million CD45+ events per sample. Lymphocyte subpopulations (CD3, CD4, CD8, CD19, NK) were analyzed using an Aquios cytometer to determine total lymphocyte counts (CD45low), which were used to calculate absolute counts of EPCs and CECs. Variables were compared between women who developed hypertensive disorders and those who did not. Correlation analyses were conducted with angiogenic markers.

Results No significant differences were observed in the percentages or absolute counts of CD34+ cells, EPCs, CECs, or lymphocyte subsets between groups across all trimesters. However, weak but statistically significant correlations ($r < 0.5$, $p < 0.05$) were found between %EPCs/%CECs and sFlt-1/PlGF ratio and PlGF levels in the 1st and 3rd trimesters.

Conclusion Although EPC and CEC levels did not differ between groups, their weak correlations with angiogenic markers suggest a potential link between vascular dysfunction and angiogenic imbalance in pregnancies at risk of preeclampsia. These findings support a multimodal approach combining vascular and angiogenic profiling for early identification of at-risk pregnancies.

Conflict of Interest No.

Title VALIDATION OF EUROFLOW ANTIBODY PANELS ON THE XF-1600 FLOW CYTOMETER **Code** 42

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Topic Hematology

Abstract

Introduction To validate the analytical performance of a comprehensive immunophenotyping strategy, the entire EuroFlow antibody panel repertoire was subjected to thorough evaluation using an XF-1600 flow cytometer within our laboratory. This rigorous assessment on the XF-1600 platform aimed to ensure the robustness and reliability of these panels for detailed cellular analysis.

Methods Following consensus sample preparation and staining procedures, including optional blocking and specific protocols for surface or intracellular antigens, data acquisition was performed on the XF-1600 flow cytometer. Quality control measures, including daily instrument QC and appropriate compensation, ensured data reliability. Subsequent analysis using dedicated software and hierarchical gating strategies allowed for the identification and characterization of distinct cell populations based on their marker expression profiles (VenturiOne, FlowJo and Infinicyt). In this study, we have used the LST, ALOT, PSCT, and SST panels for immunophenotypic analysis.

Results Comparative analysis of immunophenotyping data generated on the XF-1600 flow cytometer with results obtained using the Omnicyt and Navios platforms demonstrated a high degree of reproducibility across all three instruments. Consistent cell population identification and comparable median fluorescence intensity values for key markers were observed, indicating the reliability and robustness of the XF-1600 platform for multi-parameter flow cytometric analysis and its agreement with established cytometers.

Conclusion Our evaluation demonstrates that the XF-1600 flow cytometer provides a robust and reliable platform for comprehensive immunophenotyping using the EuroFlow antibody panels. The high degree of reproducibility observed in comparative analyses with established Omnicyt and Navios cytometers underscores the XF-1600's suitability for detailed cellular analysis.

Conflict of Interest Yes. Clare Weir, Marta Garcia-Escarp, Laura G. Rico and Tanja Tornow (Sysmex)

Title NEW FLUOROCHROMES, NEW CHALLENGES: THE FIXATION FACTOR **Code** 43

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Topic Others

Abstract

Introduction Flow cytometry enables multiparametric detection of cells and particles labeled with fluorochromes. However, fixation solutions can alter fluorescence intensity, affecting data interpretation. This study evaluates the effect of two commercial fixatives on the stability of some of the new fluorochromes currently on the market.

Methods Beads (BD CompBeads Compensation Particles Set) were labeled with a selection of tandem dyes: Brilliant Ultra Violet™ 661, Brilliant Ultra Violet™ 805 , RB545, RB780, RY610, and RB775 . They were analyzed fresh and then fixed for 30 minutes using Fix & Perm™ (ThermoFisher) or IOTest3™ (Beckman Coulter). Data acquisition was performed using a Cytek Aurora 5L cytometer at four time points: fresh, fixed at 0 h, 24 h, and 48 h. Fluorochrome stability was assessed by comparing fluorescence median intensity across conditions and spectral signature.

Results Compared to unfixed cells, we observed a variable reduction in fluorescence intensity after fixation, depending on the fluorochrome and fixative used. BUV805 showed a drastic decrease with IOTest3, reaching 0 at 24 h and 48 h, while Fix & Perm™ better preserved its signal (46534 at 24 h, 42797 at 48 h). RB545 fluorescence declined with both fixatives (Fresh: 62558 → 24 h: 35085-32 1 → 48 h: 28425-28723). RB780 remained more stable with Fix & Perm™ (Fresh: 283609 → 24 h: 235057 → 48 h: 226519) compared to IOTest3, which showed a greater decrease (Fresh: 283609 → 24 h: 26411 → 48 h: 16424). RY610 and RB775 also exhibited fluorescence loss but Fix & Perm™ maintained higher intensity (RY610: 57374 at 48 h, RB775: 112947 at 48 h). At the same time, we also observed an alteration in their spectral signature (compared to the spectral signature of unfixed reference control), leading to unmixing errors.

Conclusion Fixation significantly impacts the stability of the fluorochromes tested here, affecting both the mean fluorescence intensity and their spectral signature. In general terms, Fix & Perm™ preserved fluorescence better than IOTest3. In both cases, long-term storage of cells after fixation has a greater negative effect in the stability of the fluorochromes. The choice of the best combination fixative/fluorochrome is crucial in order to ensure optimal results.

Conflict of Interest No.

Title CD276 IMMATURE GLYCOSYLATION DRIVES COLORECTAL CANCER AGGRESSIVENESS AND T-CELL-MEDIATED IMMUNE ESCAPE **Code** 44

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Topic Solid Tumors

Abstract

Introduction Advanced-stage colorectal cancer poses a significant challenge due to treatment inefficacy. Here, we comprehensively investigated colorectal tumour's glycocalyx, focusing on protein O-glycosylation alterations and underlying glycoproteoforms as potential therapeutic targets.

Methods A retrospective analysis was conducted on a patient sample set comprising 40 formalin-fixed, paraffin-embedded (FFPE) colorectal cancer (CRC) tissues and healthy tissue sections. Immunohistochemistry was performed to screen for CD276, Tn, and STn antigens. Marker co-localization was assessed using double-staining immunofluorescence and Proximity Ligation Assays (PLA). Gene expression data from 623 colorectal adenocarcinoma (COADREAD) tumor tissues and 51 normal adjacent tissues were retrieved from the GDC database. Proteomics data from a subset of 95 COADREAD cases analyzed by mass spectrometry were reinterpreted to identify and quantify glycoproteins containing Tn or STn antigens. Cases were stratified into epithelial-like and mesenchymal-like CRC subtypes based on TCGA transcriptomic classifications and further categorized according to the presence of unglycosylated CD276 or CD276-Tn/STn glycoforms. Human CRC cell lines SW480 and SW620, which mimic CRC glycomic signatures, were glycoengineered to evaluate the functional impact of CD276-Tn/STn. These cell lines were used for functional assays, glycobiomarker discovery, and further interrogation of CD276-Tn/STn roles.

Results Tumours with unfavourable prognosis exhibited altered glycogene expression, suggesting an immature protein O-glycosylation phenotype. Their glycoprofile was characterized by reduced levels of core 3, while overexpressing Tn and, to a lesser extent, STn antigens. Glycoengineered C1GALT1 knockout cells reflecting this pattern displayed enhanced proliferation and invasion, mirroring cellular traits of aggressive tumours. Moreover, interrogation of tumours and cell models glycoproteome identified CD276 as a metastasis marker. Immature O-glycosylation conferred remarkable cancer specificity to CD276 and drove its overexpression, critical for cancer invasion and immune evasion. Additionally, it led to impaired T-cell activation mediated by CD276 and the induction of immunosuppressive cytokines and chemokines.

Conclusion These findings shed light on CD276's functional role, advocating its potential as a therapeutic target in colorectal cancer.

Conflict of Interest No.

Title PLATELET COUNTING IN MACROTHROMBOCYTOPENIC PATIENTS – COMPARISON OF FOUR METHODS **Code** 46

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Topic Hematology

Abstract

Introduction Currently, various methods are used for platelet counting in clinical practice: the impedance method (PLT-I), the optical scattering/fluorescence method (PLT-O/F), the manual microscopy counting method (MCM), and the flow cytometry (FC) method. The PLT-I counting mode is the most widely used method due to its low cost and high efficiency. However, the presence of large platelets, microplatelets, and platelet clumps can lead to falsely decreased platelet counts. The indirect flow cytometry method (dual platform), which uses red blood cells (RBC) measured by a hematology analyzer, is the reference method according to the International Council for Standardization in Hematology (ICSH). The BEST protocol, known as the direct FC method (single platform), uses beads directly measured by FC and is also recognized by the ICSH. This method has the advantage of eliminating the need for RBC and its associated interferences.

Methods The primary aim of this study was to compare the indirect FC (gold standard) with three other methods: PLT-I, MCM, and direct FC method (based on the BEST method) in thrombocytopenic patients (particular inherited thrombocytopenia). A secondary objective was to compare the results between patients with macroplatelets (mean platelet volume (MPV) >11 fL) and patients with normal size platelets across the methods. All samples were analyzed by impedance and flow cytometry (direct and/or indirect methods). The Related-Samples Friedman's Two-Way Analysis of Variance was used to compare the four methods.

Results Over a period of 10 years (January 1, 2015, to December 31, 2024), 172 samples from thrombocytopenic patients were analyzed in our laboratory. The primary clinical diagnoses included MYH9-related thrombocytopenia (25), ITGA2/ITGB3-related thrombocytopenia (28), ANKRD26-related thrombocytopenia (8), thrombocytopenia of unknown cause (45), familial thrombocytopenia without genetic characterization (4), gestational thrombocytopenia (46), Bernard-Soulier Syndrome (10), idiopathic thrombocytopenic purpura (4), Von Willebrand Disease type IIb (2). The median age of the patients was 35.4 years (ranging from 2 months to 79 years), with a sex distribution of 33 men (19.2%) and 139 women (80.8%). Platelet counts per method were as follows (median, minimum, maximum): PLT-I: 63,500 ptl/µL (4,000 to 185,000); MCM: 80,000 ptl/µL (10,000 to 174,000); Direct FC: 77,500 ptl/µL (8,000 to 162,000); Indirect FC: 80,000 ptl/µL (2,000 to 255,000). In our study, PLT-I showed a statistically significant lower platelet count compared to the other three methods. There was no statistically significant difference between the MCM, direct FC, and indirect FC methods. All four methods demonstrated a statistically significant correlation, with the strongest correlation observed between the direct FC and indirect FC methods. No statistically significant difference was found when comparing methods between the macroplatelets group and the normal size platelet group. A possible explanation could be the low number of samples with platelets having an MPV <11 fL.

Conclusion From manual phase contrast microscopy to impedance-based automated cell analyzers and more recently to flow cytometric methods, platelet counting continues to evolve, remaining a challenge in some thrombocytopenic patients, especially those with giant platelets. Flow cytometry is an accurate and reliable method. The direct FC method demonstrated consistent results in our study and may be a suitable option for laboratories without automated cell counters. Our study's results, indicating the superiority of both FC methods in patients with giant platelets, align with the existing literature. As platelet counting technologies advance, future research should assess their applicability and accuracy in specific clinical contexts to better meet the needs of practice and research, ensuring reliable and practical results.

Conflict of Interest No.

Title COMPARISON OF T-CELL CLONALITY RESULTS BY FLOW CYTOMETRY AND PCR IN PATIENTS WITH T-CELL LYMPHOPROLIFERATION AND IMMUNOPHENOTYPIC CHARACTERIZATION **Code** 47

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Topic Hematology

Abstract

Introduction An uncontrolled production of clonal T-lymphocytes may produce a mature T-cell lymphoproliferative disorder (TCLPD) or T-cell clones of uncertain significance (T-CUS) that are small clones that may exhibit immunophenotypic features resembling T-cell malignancy but often lack clinical relevance. In addition, lymphocytosis is not always clonal and it can occur in reactive conditions as infection or autoimmune diseases. In this setting, the study of T-cell immunophenotype and clonality helps in the diagnosis of TCLPD. T-cell clonality can be studied by flow cytometry (FC) using the T-cell receptor (TCR) Cβ-1 antibody, clone JOVI-1, and/or by polymerase chain reaction (PCR). This study aimed to characterize the T-cell immunophenotype and clonality by FC and to compare the results with PCR in patients with suspicion of TCLPD.

Methods We stained peripheral blood, lymph node or bone marrow samples in patients with a suspicion of TCLPD using the lymphoid screening tube (LST) at Hospital Universitario y Politécnico La Fe. When an abnormal T-cell population or an altered ratio of T CD4/CD8 was observed, an additional tube including CD2-HV450, CD45-HV500, TRBC1-FITC (JOVI-1), CD5-PE, CD3-PerCPCy5.5, CD4-PECy7, CD7-APC, and CD8-APCH7 was stained to characterize the immunophenotype and investigate T-cell clonality. The cells were acquired in a FACSCanto-II and analyzed using Infinicyt software. The PCR was performed using the Lymphoma TCR Gamma and/or TCR Beta kit, as recommended by the EuroClonality/BIOMED guidelines, and analyzed using the Genemapper software. For the comparison of both methodologies, a T-cell population was considered as monotypic by FC when TRBC1 was negative or positive in less than 5% or more than 85% of the T-cells. Using PCR, the presence of two distinct peaks or a single, clearly isolated peak indicates a clonal T-cell population. The criterion for considering one or two positive peaks is that their height must be at least 2.5 times greater than that of the adjacent peaks representing the polyclonal background.

Results "We included 16 samples (11 peripheral blood, 1 lymph node, and 4 bone marrow) from 15 patients with a suspicion of a TCLPD with a median age of 74 years (range, 22-90). Despite the limited number of cases, both methodologies showed matching results: 8 were monotypic and 8 were polytypic. Among the 8 monotypic samples, 2 (25%) were TRBC1-, while 6 (75%) were TRBC1+. The most frequent immunophenotype in the TRBC1 monotypic samples was CD3 and CD4 positive but both under expressed, a reduced or gained expression of CD5, and a reduced or absent expression of CD7. In the only CD8+ T-cell monotypic sample we found 2 immunophenotypically distinct clones by FC, identified by the different expression for CD2, CD3, CD5, and CD7, that also showed 2 different peaks by PCR. The final diagnosis for the TRBC1- monotypic patients included 1 case of CD4+ large granular lymphocytic leukemia (LGLL) and 1 case of peripheral T-cell lymphoma not otherwise specified (PTCL-NOS). Among the 5 patients with TRBC1+ monotypic samples, the diagnoses were: 1 T-CUS, 1 CD4+ LGLL, 2 PTCL-NOS, and 1 CD8+ LGLL. Of the 8 patients with TRBC1 polytypic samples, the diagnoses included 4 cases of reactive CD8+ T-cells, 2 cases of reactive CD4+ T-cells, and 2 cases of mycosis fungoides.

Conclusion The use of TRBC1 by FC can help distinguishing between clonal and reactive T-cells. FC and PCR agreed completely in detecting T-cell clonality. The ability of FC to immunophenotypically characterize small monotypic clones highlights its important role in diagnosing TCLPD. Despite the more sensitivity of FC to detect small abnormal populations compared with PCR, using both techniques is recommended to achieve more accurate and reliable results.

Conflict of Interest No.

Title VALIDATION OF A SPECTRAL CELL SORTER FOR THE MONITORING OF SAMPLES IN CLINICAL TRIALS UNDER ISO 9001 CERTIFICATION **Code** 48

Authors Names Omaira Alberquilla Fernández (1); María Luz Lozano Vinagre (1); José Carlos Segovia Sanz (1); Rebeca Sánchez- Domínguez (1)

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Topic Biotechnology and new applications

Abstract

Introduction Cell sorting of subpopulations with specific phenotypes under ISO 9001 certification is a critical component in the monitoring of gene therapy clinical trials. Vector copy number (VCN) assessment, to estimate correction efficacy, is one of the critical parameters requiring cell sorting. To validate a newly acquired spectral cell sorter, we selected one of our established protocols involving the separation of four heterogeneous cell subtypes from peripheral blood. This protocol was chosen due to its multiway sorting capability, the heterogeneity of the target populations, and the laboratory's extensive dataset available for comparison.

Methods Two independent validation studies were conducted, each using parallel samples (n = 3) from healthy donors. Equivalent operational conditions were established for both instruments, conventional and spectral cell sorters, to ensure a direct comparison. Sorting parameters were optimized to meet performance criteria across both systems, allowing for an accurate evaluation of purity, viability, and recovery metrics.

Results The purity of the studied populations in this validation followed the trend observed in the historical laboratory data, with CD3+ cells exhibiting the highest purity, followed by CD19+, CD15+, and CD14+ monocytic cells, which showed the lowest purity. Regarding instrument comparison, both systems performed adequately. Spectral cell sorter showed slightly superior results achieving higher mean purities and lower variabilities across all populations: CD3+ (99.83% [CV=0.0517%] vs. 99.1% [CV=0.7632%]), CD19+ (99.26% [CV=0.3072%] vs. 98.53%, [CV=0.5693%]), CD15+ (96.98% [CV = 1.601%] vs. 92% [CV = 6.991%]), and CD14+ (83.63%, CV = 5.741% vs. 69.02% [CV = 14.83%]). Significant differences in cell viability were observed, particularly in the lymphoid populations, with higher viability obtained in spectral cell sorter samples. No significant differences were found in recovery between the two systems.

Conclusion Our results demonstrate that both conventional and spectral systems achieve equivalent performance in terms of sorting. These results support the validation of the new spectral cell sorter for routine clinical trial sample processing. These findings confirm that both instruments are capable of effective population separation with no differences in recovery. However, the spectral cell sorter provides more consistent results, maintaining higher purity and viability across the populations studied. All authors declare that there is no conflict of interest.

Conflict of Interest No.

Title ENHANCED ANTITUMOR IMMUNITY IN POORLY IMMUNOGENIC MELANOMA THROUGH COMBINED IMILT AND PD-1 BLOCKADE **Code** 49

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Topic Immunology, Animal Sciences

Abstract

Introduction The success of immune checkpoint inhibitors (ICIs), particularly anti-PD1/PD-L1 agents, has significantly improved treatment outcomes in several cancers. However, their effectiveness is often limited in tumors with low immunogenicity—commonly referred to as "cold tumors"—due to poor T cell infiltration and an immunosuppressive tumor microenvironment (TME). Strategies capable of reprogramming the TME and promoting immune cell recruitment are essential to improve the efficacy of ICIs in such contexts. Immune-stimulating Interstitial Laser Thermotherapy (imILT) is a locoregional thermal technique that can induce immunogenic cell death, enhance antigen release, and initiate local inflammation. This may facilitate T cell priming and infiltration, rendering the tumor more responsive to immunotherapy. In this study, we evaluated the immune-modulatory effects of combining imILT with anti-PD1 therapy in a poorly immunogenic melanoma mouse model, with a focus on key immune cell populations in the tumor and peripheral compartments.

Methods C57BL/6 mice were subcutaneously inoculated with B16F10 melanoma cells and randomly assigned to four experimental groups (n=8 per group): Control, imILT alone, anti-PD1 (10 mg/kg), and imILT combined with anti-PD1 (10 mg/kg). Anti-PD1 was administered intraperitoneally three days apart, in a total of 3 treatments. imILT treatment consisted of placing a laser fiber perpendicular to the tumor, and performing for 30 minutes, at a steady-state temperature of 46ºC at the tumor margin. Upon reaching a total tumor burden of 2000 mm^3, animals were humanely euthanized. Peripheral blood of all animals was collected by intracardiac puncture and transferred into tubes containing EDTA as an anticoagulant. Tumor tissues and spleens were also collected for immunophenotyping by flow cytometry. Conjugated monoclonal antibodies were used to characterize both lymphoid and myeloid populations. The functional status of CD8+ T cells was also assessed via IFN-γ production. Data acquisition was performed using a BD FACSCanto™ II cytometer and analyzed using Infinicyt™ software. Statistical analyses were conducted using GraphPad Prism, with significance defined at $p<0.05$.

Results Treatment with anti-PD1 at 10 mg/kg and, more markedly, its combination with imILT significantly improved median survival compared to the control and monotherapy groups. Flow cytometry analysis of tumor-infiltrating immune cells revealed that the combined therapy led to increased infiltration of CD8+ T cells, natural killer (NK) cells, and conventional dendritic cells (cDCs), supporting enhanced antigen presentation and cytotoxic activity. A notable shift in CD8+ T cell phenotype was observed, with a higher proportion of effector/memory subsets and increased intracellular IFN-γ production, indicating functional activation. The CD4+/CD8+ ratio was significantly reduced in treated groups, particularly in the combination group, reflecting a shift toward a cytotoxic-dominant response. Additionally, regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), both associated with immune suppression, were significantly decreased in the tumor microenvironment and spleen in animals receiving the combined treatment. These alterations were accompanied by increased expression of activation markers such as CD44 and a reduction in naïve T cell subsets, suggesting systemic immune activation. Peripheral blood analysis reflected similar trends, with elevated frequencies of activated CD8+ T cells and reduced immunosuppressive populations. Overall, the data demonstrate that imILT and anti-PD1 act synergistically to reshape the immune landscape both locally and systemically, enhancing the antitumor response in a model typically resistant to immunotherapy.

Conclusion The combination of imILT with PD-1 blockade effectively reprograms the immune landscape in a poorly immunogenic melanoma model, promoting increased infiltration, activation, and functional maturation of effector immune cells, particularly CD8+ T cells and NK cells, while simultaneously reducing the presence of immunosuppressive populations such as Tregs cells and MDSCs. These immunological changes reflect a shift toward a more pro-inflammatory and cytotoxic tumor microenvironment, supporting the potential of this combined approach to overcome the immune resistance typically observed in "cold" tumors. The synergistic effects observed between the local thermal stimulation of imILT and systemic PD-1 inhibition highlight the value of integrating locoregional and immune checkpoint-based therapies. This strategy may offer a promising path for clinical translation, especially in tumor types that remain largely unresponsive to checkpoint inhibitors alone, and could contribute to the development of more effective, personalized immunotherapeutic regimens. Nevertheless, this combination should be further explored in other tumor types and in conjunction with additional immunotherapeutic strategies to fully understand its therapeutic potential and broaden its clinical applicability.

Conflict of Interest Yes. Clinical Laserthermia Systems AB.

Title REORGANIZATION OF THE HEMATOPOIETIC NICHE IN RESPONSE TO CHRONIC ANEMIA: NON HEMATOPOIETIC POPULATIONS AND MOLECULAR SIGNALS **Code** 50

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Topic Hematology,Animal Sciences

Abstract

Introduction Pyruvate kinase deficiency (PKD), an autosomal recessive disorder caused by mutations in the PKLR gene, is the most common cause of chronic nonspherocytic hemolytic anemia. This deficiency impairs erythroid differentiation, leading to anemic phenotypes. As in other anemic conditions, a low red blood cell count affects undifferentiated progenitors. We have demonstrated that chronic anemia due to pyruvate kinase deficiency disrupts the hematopoietic stem cell (HSC) compartment. Forced erythropoiesis increases oxidative stress and apoptosis in erythroblast pools. Simultaneously, the stem cell compartment undergoes reorganization, involving phenotypical and functional changes. Now, we aim to understand how the whole niche is affected by studying non-hematopoietic niche populations and crosstalk signals.

Methods First, we have characterized the different endothelial and mesenchymal components of the niche in healthy and PKD diseased mice using flow cytometry. Additionally, mesenchymal cells have been isolated, expanded in liquid culture, and subjected to functional studies. Finally, cytokines in peripheral blood and bone marrow from healthy and diseased mice have been quantified using flow cytometry immunoassay.

Results In the endothelial compartment, we detected a higher proportion of arteriolar endothelial cells (AECs) in the PKD bone marrow, essential for maintaining HSC quiescence, self-renewal, angiogenesis, and hematopoiesis support. Conversely, sinusoidal endothelial cells (SECs), which regulate mobilization during stress and injury, were diminished in PKD mice. Concerning mesenchymal components, we observed a decrease in CXCL12-abundant reticular (CAR) cells in PKD compared to healthy mice. At the molecular level, we identified significantly elevated concentrations of erythropoietin (EPO) and stem cell factor (SCF) in both peripheral blood and bone marrow in PKD mice. Interestingly, IL-34 levels were also markedly increased in PKD bone marrow, linking the reduction of mesenchymal CAR cells to alterations in the hematopoietic niche.

Conclusion Our findings suggest that chronic anemia due to PKD induces a reorganization of the bone marrow non-hematopoietic niche compartment, affecting both the endothelial and mesenchymal compartments. These alterations are accompanied by molecular changes in key cytokines that regulate the fate of HSCs and hematopoietic progenitors, as well as their communication with the stromal niche. This may explain in part the disrupted hematopoietic homeostasis observed in PKD.

Conflict of Interest No.

Title FLUID OVERLOAD-ASSOCIATED LARGE B-CELL LYMPHOMA: A CASE REPORT **Code** 51

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Topic Hematology

Abstract

Introduction Fluid overload-associated large B-cell lymphoma (FO-LBCL) is an emerging and distinct clinicopathology entity that was recently introduced in the fifth edition of the WHO Classification of Haematolymphoid Tumors. Unlike primary effusion lymphoma (PEL), FO-LBCL is present in immunocompetent patients with chronic fluid overload states, without solid tumor masses or lymphadenopathy.

Methods A 72-year-old man with history of chronic lung disease, obstructive sleep apnea, glaucoma, epilepsy, dementia, depressive disorder and an ex-smoker of 42 packs/year, was admitted to the hospital with dyspnea. Two weeks prior, he went to the emergency room with the same symptom, being diagnosed with a pleural effusion which was assumed to be due to pulmonary tuberculosis, starting antitubercular therapy. On admission, physical examination showed absence of respiratory sounds on the left lower lung, crackles on the right lung and an oxygen saturation of 90%. No organomegaly, lymphadenopathy or peripheral edema were found. Laboratory results revealed anemia, normal white blood cell and platelet counts, electrolytes and lactate dehydrogenase within normal ranges, and an elevated C-reactive protein of 3.66 mg/dL (normal range 0-0.5 mg/dL). The x-ray showed recurrence of pleural effusion on the left side, therefore thoracentesis and biopsies were performed.

Results Serological tests were negative for HIV, HBV, HCV, EBV and CMV. Both acid-fast staining and culture of pleural effusion were negative for Mycobacterium tuberculosis. Analysis of the pleural fluid showed a total white blood cells of 1282/µL, consisting of 77.3% mononuclear cells and an elevated adenosine deaminase of 91.8 U/L (normal range 0-30 U/L). On cytologic examination of the pleural fluid, large atypical lymphoid cells with plasmacytoid morphology were seen. These cells were positive for CD20, MUM-1, Bcl-6 and CD30 while being negative for CD3, CD10, CD38, CD138, c-Myc and HHV-8 by immunohistochemistry (IHC). Flow cytometry (FC) analysis of the pleural fluid revealed 85.2% of a monoclonal B cell population, with Kappa chain restriction, positive for CD19, CD20 and CD45, and dim for CD38. Similar cells were also observed in the pleural biopsies - large, atypical B lymphoid cells, positive for CD20 and negative for CD3, CD38, CD138, EBER and HHV-8. Pleural biopsies were also negative for Ck7, p40, calretinin and TTF-1. High-Grade Lymphoma FISH analysis was performed and was negative for MYC, BCL6 and BCL2 rearrangement. PET scan showed left pleural and pericardial thickening with hypermetabolic activity with a SUVmax 7.1 and 9.4, respectively. There were no suspicious hypermetabolic changes at other levels. The antitubercular therapy was suspended and the patient started chemotherapy with R-miniCHOP regimen. The patient completed six chemotherapy cycles, and the follow-up information showed normal blood count and blood biochemistry. TC scan showed no visible pleural effusion and the hypermetabolic thickenings on the PET scan were absence.

Conclusion FO-LBCL is a distinct entity from PEL due to its lack of HHV-8 and EBV association, occurrence in immunocompetent individuals and relatively indolent nature. Diagnosis requires high clinical suspicion, especially in cases with unexplained serous effusions and negative infectious markers. The present case highlights the unique and deceptive clinical features of this lymphoma, presenting solely with body cavity effusions without solid tumors or lymphadenopathy. Timely thoracentesis and pleural biopsies, combined with cytologic, IHC and FC analyses were pivotal for diagnosis. This patient responded well to chemotherapy, aligning with literature that FO-LBCL has a generally good prognosis when appropriately managed. Increased awareness among physicians and early diagnostic intervention are key to improving outcomes. Further research and larger case series are needed to refine treatment protocols and confirm the long-term prognosis of this emerging lymphoma subtype.

Conflict of Interest No.

Title IN-DEPTH CELLULAR IMMUNE RESPONSE PROFILING IN CRIMEAN-CONGO HEMORRHAGIC FEVER: A COMPARATIVE ANALYSIS USING COVID-19 AS A MODEL FOR PRIMARY VIRAL INFECTION **Code** 53

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Topic Immunology

Abstract

Introduction Crimean-Congo Hemorrhagic Fever (CCHF), caused by the CCHF Virus (CCHFV), is a tick-borne disease with a 35% mortality rate. Symptoms range from fever and myalgia to severe bleeding. Recent outbreaks in southern Europe, coupled with its high severity, underscore the need for a better understanding of the immune response to CCHFV and how it differs from other well characterized viral primary infections. Therefore, this study aimed to evaluate and compare the immune cell responses in CCHF and COVID-19, an extensively studied viral primary infection.

Methods We evaluated peripheral blood (PB) samples from four men diagnosed with CCHF (median age 64 years, range 53-69 years), 18 hospitalized COVID-19 patients (median age 62 years, range 50-74 years) without prior SARS-CoV-2 exposure, and 52 age-matched healthy donors (HDs). Using EuroFlow's standardized and validated antibody combinations and protocols, we employed high-sensitivity multiparametric flow cytometry to analyse >250 immune-cell populations.

Results Compared to HDs, both CCHF and COVID-19 infections resulted in decreased levels of circulating basophils, plasmacytoid dendritic cells, CD36- non-classical monocytes, central memory (CM) CD8+ T cells, CD4+ follicular helper T cells, and CM Th2 and Th17 cells, indicating a shared immune response profile to viral infections. While both infections showed a significant increase in mature (CD20- CD138+) plasma cells (PCs), CCHF patients exhibited five times the number of total PCs compared to COVID-19 patients. Notably, COVID-19 infection was consistently associated with the expansion of IgG1 and IgA1 PCs, whereas CCHF patients displayed variable immunoglobulin subclass usage. Despite this variability, CCHF patients showed a common kinetic in IgM, IgG1, and IgG3 PC production, key players in complement activation. Furthermore, CCHF patients uniquely exhibited high numbers of circulating immature neutrophils, hematopoietic precursor cells (HPCs), and CD56bright NK cells with a cytotoxic profile (Granzyme B+). A significant correlation was observed between immature neutrophil counts and PC counts (particularly IgG1, IgG3, and IgA1) and markers of tissue damage (lactate dehydrogenase, aspartate aminotransferase, and alanine transferase). These findings suggest a potential role for complement in CCHF pathophysiology, with extreme PC production potentially leading to bone marrow niche occupation and the release of immature neutrophils and HPCs into peripheral blood (PB).

Conclusion "Here we performed for the first time an in-depth evaluation of the cellular immune response in CCHF. This analysis revealed shared and unique patterns when compared with severe COVID-19, highlighting common mechanisms for viral primary viral immune responses and providing insight on the specific features of the response to infection with CCHFV. These findings contribute to our understanding of primary viral immune responses and offer a foundation for further investigation into the immunopathogenesis of CCHFV infection. Funding: CT was funded by a USAL4EXCELLENCE MSCA-COFUND fellowship (European Union's Horizon 2020 research and innovation programme; grant agreement No. 101034371), and Andrés Laguna fellowship (Junta de Castilla y León, co-financed by the Fondo Social Europeo Plus, FSE+). IC is the recipient of a Juan de la Cierva -Incorporación- IJC2020-044160-I grant (Ministry of Science and Innovation, Madrid, Spain).

Conflict of Interest No.

Title CHANGES IN TOTAL AND TRANSITIONAL B CELLS CORRELATE WITH WORSENING EDSS IN MULTIPLE SCLEROSIS PATIENTS TREATED WITH ANTI-CD20 THERAPY **Code** 57

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Topic Immunology

Abstract

Introduction Multiple sclerosis (MS) is a chronic autoimmune inflammatory disease affecting the central nervous system. Anti-CD20 therapies, such as ocrelizumab and rituximab, have demonstrated efficacy in reducing relapses and slowing disease progression by selectively depleting B lymphocytes. However, clinical responses vary among patients, and the relationship between lymphocyte subpopulations and disease progression remains unclear This study aims to analyze changes in lymphocyte subpopulations in MS patients treated with anti-CD20 therapies and explore their association with clinical progression, assessed by changes in the Expanded Disability Status Scale (EDSS) and the presence of relapses during follow-up.

Methods Patients diagnosed with MS who initiated intravenous anti-CD20 therapy without prior immunomodulatory treatment were included. Longitudinal clinical and laboratory data were collected, including EDSS scores, number of relapses, and lymphocyte counts/percentages at baseline and every 6 months during treatment (pre-infusion). Peripheral whole blood samples were analyzed every 6 months using flow cytometry to assess the following lymphocyte subpopulations: CD4+ and CD8+ T cells, B cells, and B-cell maturation stages (transitional, naïve, marginal memory, IgM memory, class-switched memory, and plasmablasts). Subpopulations were defined using CD27, IgD, IgM, CD24, CD38, and CD20 surface markers. Correlation analyses (Spearman or Pearson, depending on normality distribution) were conducted between changes in lymphocyte subpopulations and EDSS progression Linear regression models were used to quantify associations, and in cases where linear assumptions were not met or outliers were detected, robust regression using the Theil–Sen estimator was applied. Additionally, non-parametric tests (Mann-Whitney U) were used to compare lymphocyte subpopulations between patients with and without relapses, and ROC curves were generated to assess the discriminative potential in predicting clinical worsening.

Results A total of 22 patients (9 males; 13 females; median age 37.23±11.29 years) were included. All patients received intravenous anti-CD20 therapy (2 rituximab, 20 ocrelizumab) with a median follow-up of 42.27±19.55 months and an average of 8.05±3.26 doses administered. The pre-infusion total B -cell percentage and absolute count were 9.91 ± 3.65% and 188.10 ± 93.59 cells/µL, respectively. B-cell subset analysis was performed in 85 samples (those with >0.2% total B cells) revealing the following distributions: transitional (3.51 ± 2.25%), naïve (54.37 ± 17.66%), marginal memory (19.74 ± 14.43%), IgM memory (3.29 ± 4.30%), class-switched memory (18.53 ± 6.54%), and plasmablasts (0.48 ± 0.74%). An increase of 1% in total B lymphocytes from the previous measurement was associated with a 0.099- point increase in EDSS during the same period ($r = 0.203$; $p = 0.021$), as shown by linear regression. Interestingly, transitional B-cells showed a significant inverse correlation with EDSS progression (Spearman $r = -0.314$; $p = 0.043$. However, robust regression analysis (Theil–Sen) estimated a slope close to zero, suggesting that despite statistical significance, the effect size may be limited. No significant associations were found for other lymphocyte subpopulations.

Conclusion Variations in total and transitional B cell percentages correlated with EDSS changes in MS patients treated with anti-CD20 therapies, suggesting their potential as biomarkers of treatment response. Further research is needed to validate these findings and clarify their clinical relevance.

Conflict of Interest No.

Title COMBINED ANTIBODY TREATMENT AND STEM CELL MOBILIZATION AS THERAPEUTIC HEMATOPOIETIC NON-GENOTOXIC CONDITIONING **Code** 58

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Topic Hematology

Abstract

Introduction Hematopoietic stem cell transplant (HSCT) of healthy or genetically corrected hematopoietic stem cells (HSC) is an approved therapeutic approach for various hematological disorders. Currently HSCT requires highly toxic conditioning treatments which lead to significant adverse effects in treated patients. Less toxic conditioning regimens that specifically target HSCs without affecting other tissues are crucial. We have explored the synergistic approach of combining a monoclonal antibody (MoAb) treatment specifically chosen to eliminate HSCs with HSC mobilizer drugs to facilitate the access of the MoAb cocktail to them.

Methods Mice were conditioned over seven days using a combination of six monoclonal antibodies (anti-CD47, anti-c-Kit, anti-CD4, anti-CD8, anti-CD122, and anti-CD40L), following a protocol modified from George BM et al. For mobilization, mice were treated with Plerixafor, pegylated G-CSF, and/or Bio5192, alone or in combination, at different time points prior to transplantation. Following treatment, mice were transplanted with hematopoietic stem cells displaying an LSK (Lin^- $Sca\text{-}1^+$ $c\text{-}Kit^+$) phenotype. Longitudinal follow-up samples were collected over a 5-month period. Peripheral blood was analyzed regularly using a hematological veterinary analyzer. At endpoint, spleen, bone marrow, and thymus were harvested at sacrifice. Hematopoietic recovery dynamics were assessed using conventional and spectral flow cytometry with specific antibody panels.

Results We demonstrated the specific depletion of endogenous HSCs not only in wild type (WT) mice but also in two mouse models of inherited hematopoietic diseases: Rag-2 deficiency (Rag2-/-), a primary immunodeficiency disease, and Pyruvate Kinase Deficiency (PKD), a rare chronic hemolytic anemia. Our results showed a remarkable threefold increase in exogenous long-term engraftment with therapeutic efficacy and reduced risk of engraftment failure while maintaining similar kinetics of hematopoietic recovery. Our non-genotoxic conditioning treatment in a PKD model required half of the engraftment needed to reverse the anemic disease compared to conventional genotoxic treatment. This suggests notably reduced toxicity and optimal engraftment when using our novel treatment approach.

Conclusion These results constitute preclinical proof of concept for a non-genotoxic conditioning with reduced side effect toxicity that allows a functional, stable long-term and therapeutically effective hematopoietic engraftment.

Conflict of Interest No.

Title PRELIMINARY STUDY ON PD-L1 EXPRESSION IN DIFFERENT CELL LINES OF LUNG CANCER AND MELANOMA BY FLOW CYTOMETRY **Code** 59

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Topic Immunology

Abstract

Introduction Programmed Cell Death Ligand-1 (PD-L1) has emerged as a critical immune checkpoint regulator, playing a pivotal role in immune evasion by tumors. It binds to its receptor PD-1 on T-cells, resulting in the suppression of anti-tumor immune responses, which allows cancer cells to escape immune surveillance. This mechanism is especially important in the context of solid cancers like lung cancer and melanoma, where PD-L1 expression is frequently associated with poor prognosis. Recent advances in immunotherapy, particularly immune checkpoint inhibitors targeting PD-1/PD-L1, have shown promising results in treating these cancers. However, the subcellular localization of PD-L1, whether membrane-bound or intracellular, has gained increasing attention, as it may influence tumor immune escape and the effectiveness of immunotherapies. Flow cytometry is a powerful tool to assess PD-L1 expression, providing detailed insights into both surface and intracellular expression patterns. This study aims to investigate the patterns of PD-L1 expression in different non-small cell lung cancer (NSCLC) and melanoma cell lines, focusing on both surface and intracellular expression using flow cytometry.

Methods NSCLC (A549 and NCI-H460) and melanoma (A375-C5 and MeWo) cells were cultured under standard conditions. When cells reached 70-80% confluence, they were harvested using Accutase to preserve surface markers integrity. Surface PD-L1 expression was assessed using Brilliant Violet 421™ anti-human CD274 antibody, while intracellular PD-L1 was analyzed with APC-conjugated anti-human CD274 antibody. Negative controls (unstained cells and isotype controls) were included to ensure antibody specificity and define the gating strategy. Antibody titrations were performed to determine optimal concentrations. Approximately 1×10^6 cells per condition were used. For surface staining, cells were incubated with the respective antibody (60 min at 4ºC in the dark, dilution 1:20), followed by washing with FACS buffer (1200 rpm, 5 min). Cells were fixed with 2% PFA (15 min, at RT), washed again, and resuspended in sheath fluid. For intracellular staining, cells were fixed with fixation medium (15 min, at RT), permeabilized with permeabilization medium (20 min, RT), and washed with FACS buffer. Cells were stained with the respective antibody (diluted 1:80 in FACS buffer) for 30 min at 4ºC in the dark, washed, and resuspended in sheath fluid. The dual staining protocol combined the two methods. Surface staining was first performed, followed by fixation, permeabilization, and intracellular staining on the same cells. Immediately before acquisition, cells were filtered through a 0.7 µm filter. Cells were acquired on a NAVIOS Flow Cytometer (Beckman Coulter). The FACS data was analyzed using Infinicyt software (version 1.7, Cytognos SL) to determine the mean fluorescence intensity (MFI) and the percentage of positive cells.

Results Flow cytometry analysis revealed distinct patterns of PD-L1 expression across the four studied cell lines. Surface PD-L1 expression was highest in the NCI-H460 and MeWo cell lines, while the A375-C5 and A549 cells exhibited relatively lower surface expression. In contrast, intracellular PD-L1 expression was more pronounced in the A549 and MeWo cell lines compared to A375-C5 and NCI-H460, with A549 showing the most significant intracellular accumulation of PD-L1. Simultaneous detection of both surface and intracellular PD-L1 expression was pronounced in the MeWo and NCI-H460 cell lines. The dual staining protocol provided detailed insights into the localization patterns of PD-L1, revealing that the expression of this immune checkpoint protein is cell-dependent.

Conclusion This study provides important insights into the differential expression patterns of PD-L1 in lung cancer and melanoma cell lines. Our findings highlight the significance of considering both surface and intracellular PD-L1 expression when evaluating immune escape mechanisms. The data suggest that intracellular PD-L1 accumulation may be a crucial factor in immune evasion, particularly in the A549 and MeWo cell lines, where this was more pronounced. These results suggest that intracellular PD-L1 may contribute to immune escape by potentially modulating immune surveillance and response. The distinct localization patterns of PD-L1 in these cell lines further underscore the complexity of its regulation. Understanding the subcellular dynamics of PD-L1 could offer valuable insights into improving the effectiveness of PD-1/PD-L1 blockade therapies. Future studies should explore the underlying mechanisms driving intracellular PD-L1 accumulation and its impact on immunotherapy outcomes.

Conflict of Interest No.

Title IDENTIFICATION OF MONOCYTE SUBSETS IN PATIENTS WITH SUSPICION OF CHRONIC MYELOMONOCYTIC LEUKEMIA USING BY FLOW CYTOMETRY **Code** 61

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Topic Hematology

Abstract

Introduction The characterization of the monocyte subsets in peripheral blood by flow cytometry (FC) in clinical laboratories is a supporting criterion for chronic myelomonocytic leukemia (CMML) diagnosis and could help in distinguishing it from other causes of monocytosis. The use of the 6-sulfo LacNAC (SLAN) marker in a 12 monoclonal antibodies (MoAb) tube improves the identification of the intermediate and non-classical monocytes. The principal aim of this study was to compare the results of the monocytic subsets using a combination of 8 or 12 MoAb by FC.

Methods We included the patients with monocytosis, defined according with the fifth revised WHO classification (Khoury et al., 2022, Leukemia) and ICC (Arber et al., 2022, Blood) (0.5x109/L), with available paired samples analyzed using the combinations of 8 and 12 MoAb in a FACSCanto-II or a FACSLyric, respectively, in our center from May to December of 2024. We determined the percentages of the peripheral blood monocytes and their subsets, characterized by the expression of CD14, CD16, and SLAN as classical (cMo, CD14+ CD16- SLAN-), intermediate (iMo, CD14+ CD16+ SLAN-), and non-classical monocytes (nc-Mo, CD14- CD16+ SLAN+). The monocyte absolute counts were obtained from the hemogram. We used the cut off of 94% of c-Mo to consider the diagnosis of CMML, and an expression of SLAN < or = 1.7% to consider CMML with inflammatory state. Other laboratory and clinical variables were obtained from the laboratory information system.

Results A total of 24 patients with monocytosis were compared using the combinations of 8 and 12 MoAb. The median age of the patients was 72 years old (range 49-90), 16 (67%) were male. The median count of monocytes/uL was 1,260 (range, 500-21,390). The median percentage of monocytes identified with the 8 and 12 MoAb tubes were 17.95% and 18.1%, respectively, and were comparable in each paired patient samples. In all cases, a higher percentage of nc-Mo was observed when using the 8 MoAb tube compared to the 12 MoAb tube. The inclusion of the SLAN marker, that specifically identifies nc-Mo, improved the categorization of this subset. Using the 12 MoAb tube, we identified 1 patient with a final diagnosis of PTI that was initially misclassified as CMML with the 8 MoAb tube. The final diagnosis of the patients was confirmed in a bone marrow aspirate: 7 CMML (1 CMML-0, 6 CMML-1), 1 MDS with low blast count, 2 MDS with high blast count, 7 suggested but not confirmed CMML, 6 autoimmune diseases, and 1 reactive monocytosis. All the CMML had normal karyotype. The frequency of the mutations found in the 7 patients with CMML by NGS were TET2 (71%, 5/7 cases); ASLX1 and SRSF2 (43%, 3/7 cases); ZRSR2 (29%, 2/7 cases); KRAS, JAK2, SF3B1, and CBL (14%, 1/7 cases). Therefore, all the patients diagnosed as CMML were confirmed by FC and other techniques. All the patients with CMML had < or = 1.7% of SLAN expression and were classified as CMML with inflammatory state. Two of these patients had an associated autoimmune disease. Among the non-CMML patients which has a 94%), 3 of 6 autoimmune diseases, and 1 reactive monocytosis.

Conclusion The SLAN 12 MoAb tube offers a clear advantage over the 8 MoAb tube. Our findings confirm that the SLAN marker is highly valuable for identifying the i-Mo and nc-Mo subsets. Although current algorithms recommend a two-step approach for CMML diagnosis by FC, it is preferable to include the SLAN marker in all cases of monocytosis.

Conflict of Interest No.

Title ASSESSMENT OF THE PROLIFERATIVE INDEX IN B-CELL NON-HODGKIN LYMPHOMAS BY FLOW CYTOMETRY: OLD FASHIONED OR STILL IN? **Code** 62

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Topic Hematology

Abstract

Introduction B-Cell Non-Hodgkin's Lymphomas (B-NHLs) represent the most prevalent group of lymphomas in the Western world, exhibiting a spectrum of behavior from indolent forms like grade 1 Follicular Lymphoma (FL) to aggressive types such as Large B-Cell Lymphoma (LBCL) and Burkitt Lymphoma (BL). Accurate classification of B-NHL subtypes is crucial for tailored therapy; however, this can be challenging when tissue biopsies are unavailable, inadequate or non-representative. Moreover, in certain B-NHLs like FL, transformation can occur from indolent to aggressive forms, indicating a dynamic disease process, that is well correlated with the proliferative feature of the malignant B cells. Flow cytometry (FCM) offers a valuable approach for evaluating the proliferative index of malignant B cells through S-Phase Fraction (SPF) analysis, especially when using minimally invasive biopsy samples. This method enables simultaneous immunophenotyping and DNA content assessment, providing essential insights for B-NHL characterization.

Methods In a long-term study (2010 to 2020), 256 B-NHL cases diagnosed by histophatological evaluation as FL (n=111), LBCL (n=120), BL (n=20), and LBCL/BL (n=5) were analyzed by FCM. These cases included aspiration biopsies and fragments from lymph nodes, masses, bone marrow aspirates and peripheral blood. After immunophenotypic characterization, DNA content and SPF were assessed using FCM with propidium iodide or DRAQ5 staining. Data acquisition was performed on a NAVIOS Flow Cytometer (Beckman Coulter), and data analysis was made by Infinicyt (version 1.7, Cytognos SL) and ModFit LTTM software.

Results Of the 111 FL cases, 81 (73%) had a concordant diagnosis by FCM; 20 (18%) were considered FL in transformation due to the high SPF (SPF>4.7) (it should be noted that of these 20, 7 cases (35%) later revealed histological transformation to LBCL), and 10 (9%) were classified as large B-cell lymphomas (LBCL) due to the size of the malignant B cells, the high SPF and most of them had an higher DNA Index (near tetraploid DNA). Of the 120 LBCL, 103 (85.8%) had the same diagnosis and 13 were classified as FL in histological transformation (as they presented an apparently small B cell, despite the high SPF). The 4 cases classified as FL by FCM had a previous diagnosis of LBCL, despite the low SPF. Regarding the BLs, all were equally classified by the both methods. Of the 5 LBCL/LB cases, only 1 was classified as LB and the rest as LBCL. As this last group is characterized with an high degree of proliferation, the correlation with genetic findings is fundamental to reaching the correct diagnosis. Comparing the mean SPF values between the various lymphoma groups, there were, as expected, statistically significant differences ($p<0.001$): 4.26±5.37 for FL; 18.02±10.98 in LBCL; 36.46±15.94 for BL, and 26.81±10.39 for LBCL/BL group.

Conclusion Incorporating SPF determination into routine of B-NHL stratification enhances the utility of FCM, particularly with minimally invasive samples where material may be scarce and tissue architecture compromised. As highlighted in the current results, elevated proliferative indices in the B malignant populations may precede histological transformations, potentially serving as early indicator of disease progression. Thereafter, the integration of FCM findings with the other laboratory diagnostic techniques, like histology and genetics, is essential to ensure accurate diagnoses.

Conflict of Interest No.

Title BONE MARROW MICROENVIRONMENT AS A PREDICTOR OF CLINICAL STATUS IN PEDIATRIC BCP-ALL **Code** 63

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Topic Hematology

Abstract

Introduction B-cell precursor acute lymphoblastic leukemia (BCP-ALL) is the most common pediatric cancer, characterized by the malignant transformation and differentiation arrest of developing B cells in the bone marrow (BM). Although chemotherapy has significantly improved outcomes, relapse still occurs in many cases, compromising survival. Several evidence suggest that BM microenvironment has been implicated in leukemic cell survival, proliferation and resistance to therapy. The impact of the therapy on BM microenvironment and its relationship with the MRD status remains underexplored. Thus, studying changes in BM stromal cells may provide important insights into improving treatment strategies and risk stratification to predict patient outcomes. This study aims to assess the profile of BM stromal cells during treatment and their association with clinical status. The primary goal was to determine the frequency of two subtypes of stromal cells (SC), endothelial cells (EC) and mesenchymal stem cells (MSC), in the BM of pediatric BCP-LLA patients at two follow-up points and to explore their potential relationship with MRD status.

Methods A total of 72 BM samples from 47 pediatric BCP-LLA patients were considered in the study. The group was studied at two different time points, including: 47 at day +29±16 and 25 at day 78 ±15 post treatment initiation. Samples were processed according to the EuroFlow bulk-lysis SOP and stained with the EuroFlow 8-color BCP-LLA MRD panel. The samples were measured in BD FacsCanto II and BD FACSLyric flow cytometers. For data analysis, the Infinicyt software was used and the relative distribution of MSC and EC within the stromal cells (number of MSC plus EC) in BM (after excluding cell debris/doublets) were calculated. A descriptive statistical analysis was performed using SPSS version 23. The percentage of SC from all BM cells, and of MSC and EC within stromal cells, were categorized with the following cut-off values, based on the median values, at day +29 and day +78, respectively: (i) %SC> 0.042% and 0.094%; (ii) %MSC> 74.67% and 84.26%; and (iii) %EC> 22.65 % and 14.24%.

Results Using the defined cut-off values for each cellular population, the association between the percentage of SC, MSC, and EC and the presence of residual disease was assessed at days +29 and +78 using the Pearson Chi-Square test. The proportion of patients with measurable disease was similar at both time points: 29.8% at day +29 and 28.0% at day +78. Interestingly, at day +29, statistically significant associations were observed for both EC and total SC. Among patients with lower EC percentages, only 13.0% had disease, compared to 45.8% in those with higher EC percentages (p=0.014). Similarly, for stromal cells, disease was present in 16.7% of patients with lower SC percentages versus 43.5% in those with higher levels (p=0.045). At day +78, statistical analysis revealed no significant associations. However, there was a trend toward a lower disease frequency in patients with lower levels of EC, MC, and particularly SC. The percentage of disease in groups with lower versus higher cell levels was: EC (23.1% vs. 33.3%), MC (23.1% vs. 33.3%), and SC (15.4% vs. 41.7%).

Conclusion Overall, our results indicate that increased percentages of EC and SC at day +29 after therapy may be associated with the presence of MRD. Although not observed in all patients, these preliminary findings suggest that BM microenvironment profiling could provide additional information for MDR-based risk stratification in pediatric BCP-ALL. Further investigation involving larger, longer and independent cohorts of pediatric B-ALL patients is required to gain a deeper understanding of stromal cells involvement in the association with the outcome of these patients.

Conflict of Interest No.

Title CAN IMMUNOPHENOTYPIC NAÏVE/MEMORY PROFILES PREDICT PROGNOSIS IN CLL? **Code** 64

Authors Names M. Emília Sousa (1); Leonardo Maia Moço (2); Carlos Palmeira (1,3,4); Carina Faria (1); Andreia Pinto (1); Inês Lopes (1); Patrícia Maia (1); Carla Azevedo (1); Catarina Fonseca (1); Julieta Silva (1); José Mário Mariz (2); Bruno Fernandes (1); Gabriela Martins (1)

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Topic Hematology

Abstract

Introduction Chronic lymphocytic leukemia (CLL) is an adult neoplasm of small mature B-cells, characterized by high clinical heterogeneity. Determining the immunoglobulin heavy chain variable (IGHV) mutational status is essential in progressive cases requiring treatment. An unmutated IGHV status is associated with a more aggressive disease course, reduced survival, and poorer response to first-line chemoimmunotherapy, particularly due to resistance to fludarabine. Since the co-expression of CD5 and surface IgD is characteristic of naïve B-cells that have not encountered antigen in the germinal center, CLL has traditionally been considered a tumor of naïve B-cells. However, some cases lack this naïve profile, having undergone class-switch, and are therefore IGHV-mutated. Couillez et al. proposed a prognostic correlation between naïve immunophenotype (IgD+/CD27−) and unmutated IGHV, and between memory immunophenotype (IgD−/CD27+) and mutated IGHV. Our aim was to evaluate the potential prognostic value of immunophenotype in patients with suspected CLL.

Methods Nineteen consecutive samples from CLL patients collected between 2022 and 2023 were analyzed. In addition to standard diagnostic immunophenotyping, samples were stained with a monoclonal antibody panel including IgD, IgM, CD5, CD19, CD27, and CD45, and acquired on a FACSCanto II BD® flow cytometer. Data were analyzed using Infinicyt software. T cells served as the negative control for IgD expression, based on mean fluorescence intensity. Given the heterogeneity of immunophenotypes observed, cases were dichotomized based on CD27 expression. As supported by literature, IgD+/CD27+ cells were also classified under the memory phenotype. Clinical data were obtained from electronic medical records. Primary endpoints were treatment-free survival (TFS) and overall survival (OS).

Results The female-to-male ratio was 1.1:1, with a median age of 69 years (IQR: 64–76). Three patients (15.8%) were diagnosed with small B-cell non-Hodgkin lymphoma, all in advanced Ann Arbor stages. Among the remaining patients, 50% and 62.5% presented with RAI stage 0 and Binet stage A, respectively. Cytogenetic analysis was performed in nine patients, with trisomy 12 being the most common abnormality (55.6%). A 14q rearrangement was also identified. One patient progressed to Richter syndrome, manifesting as Hodgkin lymphoma. Six patients (33.3%) required treatment: four received chemotherapy with or without immunotherapy, and three were treated with BTK inhibitors. Two patients died due to progression of secondary solid tumors. Regarding B-cell immunophenotypes, three profiles were identified: naïve IgD+/CD27− (n=1; 5.3%), memory IgD−/CD27+ or IgD+/−/CD27+ (n=13; 68.4%), and "transition" IgD+/CD27+ (n=5; 26.3%). For analysis purposes, phenotypes were dichotomized as CD27− (naïve) and CD27+ (memory), with the latter comprising the majority. Within a median follow-up of 22.5 months, the 2-year TFS and OS in the CD27+ group were 58.6% and 89.5%, respectively, with both medians not yet reached. The single CD27− case had a low-risk profile, with a 13q deletion and no indication for treatment.

Conclusion In conclusion, the absence of reached medians for TFS and OS in patients with a memory-like phenotype aligns with the generally favorable prognosis of mutated IGHV cases. Interestingly, the sole patient with a 14q rearrangement exhibited both IgD and CD27 expression, highlighting that these markers are not mutually exclusive. This "transition" phenotype warrants further investigation, particularly regarding its association with IGHV mutational status. Limitations of the study include the small sample size, considerable immunophenotypic variability, and the lack of available IGHV mutation data to correlate with flow cytometry findings.

Conflict of Interest No.

Abstracts

Publication

Title ADVANCING CELLULAR ANALYSIS: A COMPREHENSIVE WORKFLOW USING LABEL-FREE SPECTRAL FLOW CYTOMETRY AND IMAGING **Code** 1

Authors Names Laura Ferrer (1); Moen Sen (1); Aaron Middlebrook (1); Shivani Upadhyaya (2); Scott Bornheimer (1); Melissa S. Roth (2); Stephanie Widmann (1); Aaron Tyznik(1)

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Topic Biotechnology and new applications

Abstract

Introduction Recent advancements in flow cytometry, particularly with the integration of BD CellView and BD SpectralFX Technologies in the BD FACSDiscoverTM platform, have significantly enhanced cell analysis and sorting capabilities. This innovative combination merges spectral flow cytometry with real-time spatial and morphological insights, utilizing imaging parameters and raw fluorescence data. This approach facilitates new biological discoveries in challenging samples with high autofluorescence, such as green algae.

Methods In this study, green algae samples were analyzed using the BD FACSDiscoverTM platform, which incorporates BD CellView and BD SpectralFX Technologies for real-time sorting. The system captures autofluorescence, images and imaging parameters, providing detailed spatial and morphological insights. Data analysis was conducted using FlowJo v10.10, with concatenated FCS files processed through the T-Rex (Tracking Responders EXpanding) plugin for targeted clustering. UMAP dimensionality reduction and clustering (KNN and DBSCAN) were performed using all imaging and fluorescent parameters. Clusters were annotated with Cluster Explorer and Marker Enrichment Modeling (MEM), and the HyperFinder algorithm was employed to develop optimal gating strategies. These strategies were then applied back to the instrument to visualize and verify cell phenotypes.

Results While high autofluorescence samples have typically been a challenge for the cytometry community, this study used a label-free methodology to characterize photoautotrophic, mixotrophic and heterotrophic algae. Photoautotrophic, mixotrophic and heterotrophic algae clustered separately, with more intra-sample heterogeneity observed in the autotrophic algae. Clusters were identified by their unique morphology and autofluorescence. Novel gating strategies generated through HyperFinder allowed the unique clusters to be identified on the instrument and associated images visualized on the experiment page, enabling phenotypic validation of the clusters identified by size, morphology, and chlorophyll expression of the cells. Importantly, the gating strategy defined by HyperFinder efficiently utilizes fluorescent and imaging parameters to identify unique subpopulations that can be used as sort gates for downstream functional studies of these populations.

Conclusion This study presents a comprehensive workflow for the unsupervised characterization of cellular heterogeneity using label-free spectral flow cytometry and imaging parameters. This method is applicable to various sample types, including those with high autofluorescence, providing researchers with powerful tools to explore previously unanswerable biological questions.

Conflict of Interest Yes. Laura Ferrer, Moen Sen, Aaron Middlebrook, Scott Bornheimer, Stephanie Widmann and Aaron Tyznik are employees of Becton Dickinson, Inc., the manufacturer of the BD FACSDiscover S8™ Cell Sorter used in these studies.

Title ALL-B DEVELOPMENT AFTER LENALIDOMIDE TREATMENT OF MULTIPLE MYELOMA: A SERIES OF 5 CASES **Code** 30

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Topic Hematology

Abstract

Introduction Lenalidomide is an immunomodulatory drug used for the treatment and maintenance of multiple myeloma (MM), among other diseases. Lenalidomide modifies the substrate specificity of the CRBN-CRL4 E3 ubiquitin ligase, targeting IKZF1 and IKZF3, which are essential transcription factors in MM. This mechanism may be implicated in the development of secondary myeloid malignancies. Emerging evidence also suggests a potential association between lenalidomide maintenance therapy in MM and B-cell acute lymphoblastic leukaemia (B-ALL). Some studies propose that B-ALL may arise as a clonal haematopoiesis of indeterminate potential, independent of the primary MM.

Methods We report five cases of B-ALL diagnosed in patients with MM following lenalidomide exposure at our institution between 2018 and 2024. Our analysis focused on demographic data, clinical history, flow cytometry (FC), conventional and molecular cytogenetics, and treatment regimens. Additionally, we compared clinical findings with published literature to evaluate similarities in disease characteristics, management strategies, and patient outcomes.

Results The median age at MM diagnosis was 60.5 years (range 52-69). At diagnosis, the plasmatic cell (PC) count by FC ranged from 11% to 32%. All patients were CD56+, with two patients being CD117+, two CD28+, and three CD27+. All patients showed heterogeneous CD81 expression. FISH studies did not reveal significant differences compared to other MM cases studied in our centre. However, two patients exhibited poor prognosis markers: t(4;14) and 1q duplication. Treatment regimens for all included bortezomib and dexamethasone, some received cyclophosphamide. Lenalidomide was introduced at different stages during treatment for each patient. All patients undergoing HSCT were conditioned with melphalan. On average, 42 months (range 24-60) elapsed from lenalidomide initiation to B-ALL development. At B-ALL diagnosis, four patients presented with significant cytopenias. The median blast count by FC was 57% (range 29-85%). All were CD34+ cIgM- CD58+ and TdT+, with four patients being CD10+, one CD33+, and one CD66c+. FISH studies were negative for BCR::ABL1 (Philadelphia chromosome), E2A::PBX1 t(1;19), KMT2A rearrangements, ETV6::RUNX1 t(12;21), and BCR::ABL t(9;22). Genetic testing was available for only one patient, showing hypodiploidy, a pathogenic TP53 mutation, and IKZF1/CDKN2A/B deletions. All patients succumbed to the disease, with a median survival of 11.5 months (range 1-22).

Conclusion Although lenalidomide benefits MM treatment, healthcare professionals must be aware of the increased risk of secondary malignancies associated with its use. The time from lenalidomide initiation to B-ALL onset varies widely in the literature, suggesting that treatment duration may not be the sole determining factor. This highlights the growing need for comprehensive molecular studies to deepen our understanding of the pathophysiology and classification of this emerging entity. In our cohort, the absence of complete genetic data limits direct comparisons; however, TP53 and IKZF1 alterations were identified in the patient who underwent genetic analysis. Compared to the literature, a key difference in our patients was the higher B-ALL blast count by FC at diagnosis (29-85%) compared to previously reported cases (3-30%). Notably, regression of an abnormal immature B-cell population with a B-ALL immunophenotype has been observed following lenalidomide withdrawal, emphasizing the need for early identification of those abnormal blasts. This suggests the necessity for closer monitoring, including FC characterisation of B cell precursors. Defining the optimal timing and methodology for such monitoring remains a key area for further research. Communication funded by Project GR24186 through the European Union's "European Regional Development Fund"

Conflict of Interest No. All authors declare that they have no conflicts of interest.

Title COMPARISON OF LYMPHOCYTE SUBSET RESULTS BETWEEN EDTA AND SODIUM-HEPARIN TUBES AND SAMPLE STABILITY IN DEDICATED FLOW CYTOMETRY **Code** 39

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Topic Accreditation and management

Abstract

Introduction Screening assays for suspected immunodeficiency frequently require multiple tests involving different blood collection tubes. Among these assays, flow cytometry-based analyses are commonly performed, including basic lymphocyte subset enumeration and functional assessments. EDTA tubes are currently used for immunophenotyping but are unsuitable for most immune functional assays that require calcium. Moreover, pediatric patients often have limited vascular access, making it crucial to minimize the number of tubes required. This study aims to compare lymphocyte subset results obtained from sodium-heparin (NaHep) and EDTA tubes, as well as evaluate sample stability analyzed within a dedicated flow cytometry setting.

Methods A total of 35 healthy subjects (HS) participated in this study. Blood samples were simultaneously collected into two different tubes (EDTA and NaHep). All samples were transported to our laboratory and processed within 2 hours of collection using the AQUIOS CL flow cytometer (Beckman Coulter). Five out of the 35 samples were also processed at 24, 48, and 72 hours post-sampling. Both the relative frequencies and absolute counts of different lymphocyte subsets were automatically analyzed using AQUIOS software. Spearman correlation, Bland-Altman analyses, and linear regression models were applied to each parameter.

Results The correlation of the analyzed lymphocyte subsets ranged from 0.971 to 0.993 ($p < 0.001$). The strongest correlation was observed in NK frequency [mean difference -0.3626, 95% CI (-2.23 to 1.50)], while the weakest correlation was found in CD19 enumeration [mean difference 2.45, 95% CI (-33.54 to 38.45)]. Although no significant differences were observed in the regression models between container type and time, a trend indicates that EDTA tube values are more time-sensitive compared to those obtained from the NaHep tube.

Conclusion The relative and absolute counts of lymphocyte subsets obtained from EDTA and NaHep tubes demonstrated comparable results. In cases of challenging venous access, particularly in young children, a single blood collection tube may suffice for immunodeficiency screening by flow cytometry. Regardless of the tube type, results remain comparable for up to 48 hours.

Conflict of Interest No.

Title PLASMABLASTIC LYMPHOMA VERSUS PLASMABLASTIC MYELOMA: A DIAGNOSTIC CHALLENGE IN THE ERA OF FLOW CYTOMETRY **Code** 52

Authors Names Verónica Casado Cabanillas (1); Karla Susana Calzadilla Román (1); Ana María Valderrama Rosa (1); Diana Carolina Patiño Culma (1); Luz Gema Román Molano (1); Víctor Higuero Saavedra (1); María Helena Bañas Llanos (1); Juan Miguel Bergua Burgués (1)

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Topic Hematology

Abstract

Introduction The differentiation between plasmablastic lymphoma (PBL) and plasmablastic myeloma (PBM) represents a significant diagnostic challenge due to overlapping morphological and immunophenotypic features. Flow cytometry (FCM) has become a key tool for discerning cell populations, especially in atypical presentations. We present the case of a non-immunocompromised male patient with clinical and laboratory characteristics that illustrate the complexity of this differential diagnosis.

Methods A 64-year-old male with no relevant medical history was admitted in February 2025 due to a respiratory infection. Computed tomography (CT) revealed a 7 cm extrapulmonary mass in the left thoracic wall with rib destruction, lytic lesions in the sternum, a 12.5 × 8 cm mesenteric mass, and multiple retroperitoneal lymphadenopathies. Cranial CT showed a 4 × 3.5 cm parieto-occipital lesion with an associated soft tissue component. Blood tests showed a progressive decline in hemoglobin over recent years, with mild anemia at presentation (Hb 11.9 g/dL), without hypercalcemia or renal failure. Serum protein electrophoresis identified a monoclonal IgA kappa component of 1.1 g/dL. β2-microglobulin was elevated (5.4 mg/L). LDH was normal. The patient did not report constitutional symptoms. FCM studies were conducted on bone marrow (BM) samples and a biopsy of the parieto-occipital lesion, complemented by evaluation of the proliferative index (Ki-67), immunohistochemistry, and cytogenetic analysis (still pending in BM).

Results FCM analysis of the BM revealed a population of approximately 30% with a plasmacytic phenotype (CD38+, CD138+, CD19–, CD20–) and kappa light chain restriction. Two subpopulations were identified: Population A (11.5%): CD45++, CD10+, CD28++ (homogeneous), CD79a+, CD56–, CD27–. Population B (17%): CD45–, CD56+ (50%), CD27+, CD81 dual, CD28+d (heterogeneous). The biopsy of the parieto-occipital mass showed infiltration by lymphoid cells, some with plasmablastic differentiation. FCM of this sample revealed an identical immunophenotype to BM Population "A", with no evidence of two populations. The Ki-67 index was 70%. EBER study and serologies for HIV and Epstein-Barr virus (EBV) were negative.

Conclusion This case illustrates the complexity of this diagnostic dilemma, with evidence of two distinct clonal populations in the BM with a plasma cell phenotype: one with mature characteristics and the other with more "lymphoid" features by FCM, the latter being predominant in the extramedullary mass. Although the presence of a monoclonal gammopathy, EBV negativity, absence of immunodeficiency, and a proliferative rate below 80% could suggest a transformation to plasmablastic myeloma, the atypical clinical presentation—marked by prominent nodal involvement and a mesenteric mass—and the absence of classic myeloma symptoms (as lytic lesions can occur in both entities) provide conflicting data that challenge the traditional classification between PBM and PBL. In this context, FCM emerges as a valuable diagnostic tool, allowing for precise identification of cell subpopulations and determination of immunophenotypic profiles that, together with radiological and clinical findings, point toward a divergent neoplastic transformation. Integrating these multiple levels of analysis is crucial to elucidate the nature of the pathological process, optimize diagnosis, and consequently define the most appropriate therapeutic strategy for these hard-to-categorize cases.

Conflict of Interest No.

06.
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