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ABSTRACT BOOK

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ORAL COMMUNICATIONS

HEMATOLOGY 1

STAT3 and STAT5b mutations in T/NK chronic lymphoproliferative disorders of large granular lymphocytes (LGL): relationship with immunophenotypic and clinical features

Noemi Muñoz-Garcia[1,2,3,4], María Jara-Acevedo[1,2,3,4], Carolina Caldas[1,2,3,4], Paloma Bárcena[1,2,3,4], Antonio López[1,2,3,4], Noemi Puig[2,3,5], Miguel Alcoceba[2,3,5], Neus Villamor[6], Paula Fernandez[7], Marcos Gonzalez[2,3,5], Alberto Orfao[1,2,3,4], Julia Almeida[1,2,3,4]

[1]Cancer Research Center [IBMCC, CSIC-USAL], Salamanca, Spain, [2]Institute for Biomedical Research of Salamanca (IBSAL), Salamanca, Spain, [3]Center for the Biomedical Research Network in Oncology (CIBERONC), Salamanca, Spain, [4]Department of Medicine and Service of Cytometry (NUCLEUS), Salamanca, Spain, [5]Department of Hematology (University Hospital of Salamanca), Salamanca, Spain, [6]Department of Pathology, Hematopathology Unit (Hospital Clínic), Barcelona, Spain, [7]Institut für Labormedizin (Kantonsspital), Aarau, Switzerland

Introduction:

T-cell LGL leukemia (T-LGLL) and chronic lymphoproliferative disorders (CLPD) of NK-cells (CLPD-NK) are recognized by the WHO classification as mature (rare) neoplasms that usually show an indolent clinical course. Despite this, both diseases are often associated with autoimmune disorders and even in some cases may evolve into aggressive leukemias. Recently, somatic *STAT3* and *STAT5b* mutations have been found in a subgroup of T-LGLL (particularly in $CD8^+$ T-LGLL) and CLPD-NK, its systematic utility in clinical settings remaining uncertain. We aimed to analyze the frequency and type of somatic mutations in the *STAT3* and *STAT5b* genes in all subgroups of clonal LGL expansions –including T□□-LGLL and T□□$CD4^-CD8^{-/lo}$-LGLL, among other categories not yet analyzed for the presence of *STAT3* and *STAT5b* mutations–, and to relate these findings with both the phenotype of clonal cells and clinical behavior, to better establish its diagnostic and prognostic utility.

Methods:

STAT3 and *STAT5b* genes were sequenced in 159 populations of FACS-sorted cells (previously phenotyped by multiparametric flow-cytometry using EuroFlow protocols and panels), from patients with 93 monoclonal and 7 oligoclonal LGL expansions, 19 non-cytotoxic T-CLPD and 40 polyclonal T-cell populations.

Results:

STAT3 and *STAT5b* somatic mutations were detected in 31/93 populations from patients with clonal LGL expansions (33%), all but one in the *STAT3* gene (Y640F was the most frequent mutation, among others previously described). Furthermore, one not previously described mutation was found (K658F). According to the cell-lineage involved, mutations were present in 34% of T□□$CD8^+$-LGLL, 38% of CLPD-NK, 8% of T□□$CD4^+CD8^{-/+lo}$-LGLL, 100% of T□□$CD4^+CD8^+$-LGLL, 50% of T□□$CD4^-CD8^-$-LGLL and 44% of T□□-LGLL. *STAT3* mutation was also detected in 1/17 non-cytotoxic T-CLPD, but in none of polyclonal cytotoxic T-cell populations. There were not

differences between the cell-lineage and the type of mutation; likewise, the phenotype of clonal cells largely overlapped between mutated *vs.* non-mutated cases. Interestingly, two patients who had two or more LGL expansions from different cell-lineages showed *STAT3* mutations in at least two of them, which supports monoclonality. In 27/28 mutated cases with clinical information available, patients showed a significantly higher incidence of autoimmune disorders (*vs.* non-mutated monoclonal LGL expansions), further supporting the prognostic interest.

Conclusions:

Our results support the utility of the *STAT3* and *STAT5b* mutations study for clonality assessment and prognostic evaluation in T-LGLL and CLPD-NK; additionally, these findings suggest that similar activation pathways might be involved in the pathogenesis of LGLL, regardless of the cytotoxic cell-lineage involved.

High frequency of monoclonal B-cell lymphocytosis among Japanese descendants living in Brazil

Mariane Faria-Moss[1], Mihoko Yamamoto[1], **Ignacio Criado[2]**, Celso Arrais-Rodrigues[1], Caio Perez-Gomes[3], Maria de Lourdes Chauffaille[1], Matheus Vescovi Gonçalves[1], Efstathios Koulieris[2], Eliza Y Sugano Kimura[1], João Bosco-Pesquero[3], Julia Almeida[2], Alberto Orfao[2]

[1]Division of Hematology, Universidade Federal de São Paulo (EPM/UNIFESP), São Paulo, Brazil, [2]Cancer Research Centre (IBMCC, USAL-CSIC), Department of Medicine and Cytometry Service (NUCLEUS), University of Salamanca, CIBERONC and IBSAL, Salamanca, Spain, [3]Department of Biophysics, Universidade Federal de São Paulo (EPM/UNIFESP), São Paulo, Brazil

Introduction:

Chronic lymphocytic leukemia (CLL), the most common adult leukemia in Western countries, is much less frequent in Asian populations, even among Asians living in Western countries. Currently, it is known that virtually all CLL are preceded by a monoclonal B-cell lymphocytosis (MBL), categorized into high-count (MBL^{hi}) or low-count (MBL^{lo}) MBL, based on the presence of ≥ or <0.5 CLL-like clonal B cells/L, respectively. While CLL-like MBL^{lo} is detected in 7-14% among individuals older than 40y in Western countries, the prevalence of CLL-like MBL^{lo} among Asians is currently unknown. We aimed to determine the frequency and characteristics of CLL-like MBL^{lo} among Japanese individuals living in a Western environment.

Methods:

CLL-like MBL was investigated in 258 adults (median age: 66y, range 40y-96y) Japanese descendants, without race miscegenation, living in Sao Paulo (Brazil). Peripheral blood (PB) samples (2 mL/case) were stained using high-sensitive 8-color flowcytometry to screen -on at least $5x10^6$ leukocytes- for the presence of CLL-like cells and to analyze the major leukocyte populations. CLL-like cells were sorted for CLL-associated cytogenetic abnormalities (n=8) and *IgHV* mutational status (n=6) studies. The study was approved by the local Ethical Committee.

Results:

CLL-like cells were detected in 27/258 subjects (11%; 15% males and 7% females, p=0.05). Median clone size was 0.12/µL (range: 0.03-20/µL), larger in men vs. women (p=0.04). The frequency of MBL progressively increased with age, from 3.8% among subjects aged 40-59y to 22% among those ≥80y (p=0.02). The clonal sizes also increased by age (p=0.04). Almost half of MBL cases were biclonal (13/27; 48%); no significant differences were found in clonal sizes between monoclonal and biclonal cases. Males tended to be more represented among MBL^{lo} (16/27; 59%) vs non-MBL (108/258; 42%) individuals (p=0.06). PB T cells (p=0.05**)** and NK cells (p=0.002) were higher in MBL^{lo} vs. non-MBL cases. None of the 8 CLL-like clones studied carried CLL-associated cytogenetic abnormalities, and 4/6 cases displayed mutated *IgHV*.

Conclusions:

The frequency of CLL-like MBL^{lo} among Japanese descendants living in Brazil is similar to Western countries. The lower clonal size observed in this series, together with a higher rate of biclonality and the absence of cytogenetic alterations (vs clones observed in Western populations) may reflect earlier stages of MBL^{lo} development. Further studies in both Asian and Western populations are required to better understand the mechanisms leading to CLL-like cell emergence (similar in both populations) and progression to CLL (almost restricted to Western countries).

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Post HSCT survival in Acute Myeloid Leukemia adult patients, according to the pre transplant Minimal Residual Disease by Flow Cytometry

Maura RV Ikoma-Colturato[1], Anderson Simione[1], Camila Bertolucci[1], Joana Conti[1], Iago Colturato[1], Fernanda Franceschi[1], Fernanda Rodrigues[1], Ederson Mattos[1], Mair Souza[1], Vergilio Colturato[1]
[1]*Amaral Carvalho Hospital, Jau, Brazil*

Introduction:

MRD evaluation by flow cytometry (MFC) in AML has low sensitivity, but is useful for prognosis, inclusive before hematopoietic stem cell transplantation (HSCT).

Methods:

Data basis from our HSCT service was used to evaluate the overall survival (OS), the disease free survival (DFS) and the cumulative incidence of recurrence (CIR) of 194 AML adult patients (18 to 60 years of age), submitted to the 1st allogeneic HSCT, between January 2008 and June 2017, who did MRD by MFC before transplantation. The median follow-up was 1 year. The strategies of MFC in 4 and 8 colors were the detection of the leukemia-associated phenotypes (LAIPs), as well as the "different from normal" phenotypes, even if the initial phenotype was unknown. The MRD cut-off for positivity and negativity was > or < 0.01%. Patients were classified into three groups: clinical remission with positive MRD (CR/MRD+), clinical remission with negative MRD (CR/MRD-) and active disease (AD). SPSS (IBM) ® software was used for Kaplan Meier survival curves analysis.

Results:

OS (p=0.004) and DFS (p = 0.002) were 49% and 47% in the CR/MRD- group (n=130); 23% and 21% in the CR/MRD+ group (n=36); 13% and 20% in the AD group (n=28) (p=0.09). The results remained, selecting patients with myeloablative conditioning regimen (n=178): OS (p=0.009), SLD (p=0.006) and AD (p=0.35). In order to evaluate the influence of the MFC sensitivity on the results, the patients receiving myeloablative regimen and ≤ 2RC evaluated with 8-color (n=76) and 4-color (n=87) MRD were studied separately. At 3 years, OS was 62%, 28% and 48% (p=0.05), DFS was 62%, 34% and 38% (p=0.04) respectively for CR/MRD-, CR/MRD+ and AD groups, with no difference of CIR among the groups (p=0.2). In 4-color MRD patients, OS was 49%, 43% and 22% (p=0.01), DFS was 45%, 43% and 22% (p =0.01) respectively, without significance of the CIR (p=0.08).

Conclusions:

Despite the low sensitivity, MRD by MFC is a predictive factor of recurrence post HSCT in AML patients, considering the post-transplant recurrence rate in patients without MRD detectable before it. We observed differences between evaluation of 8 color MRD comparing with 4 colors approach, indicating an increase in the sensitivity of the method, influenced by the different strategies for MRD assessment and by the continued development of expertise in MFC data analysis. The results corroborate with the data that there is no prognostic difference between patients transplanted with active disease or with MRD+.

MICROBIOLOGY AND PLANT SCIENCES

Short-term effects of the fragrance tonalide on the microalga Chlamydomonas reinhardtii

Marta Esperanza[1], Marta Seoane[1], Carmen Rioboo[1], Ángeles Cid[1]
[1]*Laboratorio de Microbiología, Facultad de Ciencias, University of A Coruña, A Coruña, Spain*

Introduction:

Levels of contaminants in the aquatic ecosystem have increased alarmingly across the world over the past few decades. Personal care products (PCPs) are a diverse group of emerging compounds used in all kind of cosmetic formulas such as soaps, lotions, toothpastes, fragrances or sunscreens. One group of important aquatic contaminants widely used as additives in PCPs are synthetic musk compounds, especially polycyclic musks such as tonalide (6-Acetyl-1,1,2,4,4,7-hexamethyltetralin). Tonalide not only contributes to give them a characteristic and pleasant scent but it also serves to maintain the integrity of the products. In ecotoxicology, microalgae are of primary interest when addressing the potential adverse effects of pollutants, due to their characteristics and position in the aquatic food web.

The aim of the present study was to evaluate the potential acute toxicity of tonalide, included in the main classes of PCPs, on the freshwater microalga *Chlamydomonas reinhardtii*.

Methods:

Cultures of *C. reinhardtii* were exposed for 24 h to several tonalide concentrations (expressed as mg L^{-1}) that were chosen taking into account the 96 h EC_{50} value for growth: 1.25 (half EC_{50} value), 2.5 (EC_{50} value) and 5 (double EC_{50} value). The effect of the fragrance was quantified on different parameters using different flow cytometry protocols: growth rate, chlorophyll *a* fluorescence, cell viability, cellular metabolic activity, oxidative stress, mitochondrial and cytoplasmic membrane potentials and intracellular pH.

Results:

Results in the present study showed that tonalide caused dose-dependent alterations on the cellular physiology of *C. reinhardtii* after 24 h of exposure. Growth rate, chlorophyll *a* fluorescence, viability cells, metabolic activity (esterase activity) decreased significantly, whereas cytoplasmic and mitochondrial membrane potentials, ROS levels and intracellular pH increased significantly in cultures exposed to tonalide.

Conclusion:

It can be concluded that the model organism used and the toxicological parameters evaluated are suitable to assess the toxicity of this emerging contaminant. This research supports the need of establishing environmental quality standards (EQS) for polycyclic synthetic musk based on toxicity testing with key aquatic organisms, as well as identifying and reducing the different input sources to the environment.

This work was carried out with the financial support of the Spanish “Ministerio de Economía, Industria y Competitividad” (CTM2017-88668-R).

Differential cytotoxic response of two microalgal species to the anti-inflammatory drug diclofenac

Sara Martínez-Dopico[1], Marta Esperanza[1], Marta Seoane[1]
[1]*Laboratorio de Microbiología, Facultad de Ciencias, Universidade da Coruña, Campus da Zapateira s/n, 15071, A Coruña, Spain*

Introduction:

Diclofenac is a nonsteroidal anti-inflammatory drug commonly used throughout the world. Its global ubiquity in various environmental compartments, including aquatic environments, and its potential toxicity to several organisms from different trophic levels, make diclofenac an emerging environmental pollutant. Therefore, it seems necessary to evaluate the environmental risks of this compound, including its effects on non-target organisms, such as microalgae, the main primary producers of aquatic ecosystems. The aim of this study was to evaluate the potential cytotoxic effects induced by increasing concentrations of diclofenac on the freshwater microalgae *Chlamydomonas reinhardtii* and on the marine diatom *Phaeodactylum tricornutum*.

Methods:

Control cultures and cultures exposed to different concentrations of diclofenac (0.25, 0.50, 0.625 and 0.75 ng cell $^{-1}$) were established under controlled light and temperature conditions. The potential cytotoxic effect of this drug on the two microalgal species was analysed by flow cytometry after only 24 hours of exposure. In addition to the traditional growth endpoint, several morphological and physiological parameters such as relative cell size and complexity, chlorophyll *a* fluorescence, cellular viability and metabolic activity, intracellular pH, reactive oxygen species (ROS) content and cytoplasmic membrane potential were evaluated as early potential biomarkers of cytotoxicity.

Results:

The results showed that diclofenac is toxic for the two microalgal species tested, but they showed a different sensitivity and cytotoxic response. Although growth was not significantly affected in *C. reinhardtii,* even at the highest concentration tested, a decrease in the metabolic activity of the exposed cultures was observed with respect to the control ones. In *P. tricornutum* cultures exposed to diclofenac, a decrease in growth, cellular autofluorescence and complexity, an increase in intracellular pH and cytoplasmic membrane depolarization were detected. Neither of the species showed alterations on cell size, viability and ROS content. Metabolic activity, using the fluorescein diacetate cytometric assay, could not be studied in *P. tricornutum*.

Conclusions:

Obtained results suggest that the presence of diclofenac in the environment can damage the phytoplankton community causing an imbalance in marine and freshwater ecosystems since, in one way or another; both species were affected, being *P. tricornutum* the most sensitive. Despite the changes observed at the cellular level, the results suggest that both species are able to overcome the stress produced by maintaining their cellular viability and controlling the intracellular concentration of reactive oxygen species.

This work was carried out with the financial support of the Spanish “Ministerio de Economía, Industria y Competitividad” (CTM2017-88668-R)

Can Flow Cytometry be an alternative method in Extracellular Vesicles isolation?

André Mozes[1], Oksana Sergeeva[2], Valérie Glutz[1], Loïc Tauzin[1], Miguel Garcia[1]
[1]Flow Cytometry Core Facility, EPFL, Lausanne, Switzerland, [2]Global Health Institute, SV, EPFL, Lausanne, Switzerland

Introduction:

Extracellular Vesicles are membrane contained vesicles released from prokaryotes to higher eukaryotes and even plants. The fact that the information that this EVs carry can influence the recipient cell function is maybe their most important characteristic, as they are able to transport mRNA and miRNA, covering large distances.

Although EVs have been isolated from different sources, if we take in account all the heterogeneity inside the EVs world, we do not have yet a robust and effective method to isolate the different types of EVs.

In order to answer to some of our users and to contribute to the development of the EVs global knowledge, we decided to test if we could use a non-dedicated equipment from our multi-user core facility, to isolate this vesicles or at least to have a better understanding of our limitations in this field.

Methods:

Retinal pigment epithelial 1 (RPE-1) cells (RRID: CVCL_4388) were used during this study, as well as Apogee Mix beads (Cat#1493) from Apogee Flow Systems.

Exosomes sort was performed using a 4-laser MoFlo Astrios EQ from Beckman Coulter.
The Nano Sight NS300 from Malvern Instruments Ltd™ was used to evaluate the beads sorted population's size.
The transmission electron microscopy (TEM) data was acquired using a Tecnai™ Spirit Bio TWIN® with 120KV electron microscope. For LC-MS/MS analysis, resuspended peptides were separated by reverse phase chromatography on a Dionex Ultimate™ 3000 RSLC nano UPLC system connected in line with a Qexactive HF® from Thermo Fisher Scientific™
Data analysis was done using FlowJo®v10.1, Scaffold4® and String®.

Results:

The characterization of our FACS sorted EVs by TEM, and LC – MS/MS, showed that our FACS-sorted EVs were consistent with real exosomes regarding their size and composition.

Interestingly, 9 out of the 35 identified proteins belong to the top 100 exosomes proteins listed on the exocarta.org website. Furthermore, EXO1, ANXA2 and GAPDH belong to the top 10 of the exosomes related proteins according to the same website.

Conclusion(s):

With the instrument configuration and sorting strategy chosen, we were able to successfully sort extracellular vesicles, most probably exosomes. At the same time, we have shown that you don´t need to have a dedicated instrument inside a core facility to be able to sort EVs. It is possible, just by applying minor changes that would not have significant interference in a multi-user core facility.

IMMUNOLOGY 1

EuroFlow-PERISCOPE 14-color combination for the identification of multiple functionally-relevant CD4+ T-cell subsets

Vitor Botafogo[1,2], Martín Pérez-Andres[1], Paloma Bárcena[1], María Jara-Acevedo[1], Daniela Damasceno[1], Elena Blanco[1], Veronique Corbiere[3], Françoise Macart[3,4,] Cecile van Els[5], Annieck Diks[6], Magda Berkowska[6], Jacques J.M. van Dongen[6], Julia Almeida[1], Alberto Orfao[1]
[1]Cancer Research Centre (IBMCC, CSIC-USAL), Cytometry Service (NUCLEUS), Department of Medicine (University of Salamanca), Institute of Biomedical Research of Salamanca (IBSAL) and CIBERONC, Salamanca, Spain, [2]Clinical Medicine Postgraduate Program, Faculty of Medicine, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [3]Laboratory of Vaccinology and Mucosal Immunity, Université Libre de Bruxelles , Brussels , Belgium, [4]Immunobiology Clinic, Hôpital Erasme, Brussels, Belgium, [5]Centre for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM) , Bilthoven, The Netherlands, [6]Department of Immunohematology and Blood Transfusion (IHB), Leiden University Medical Center, Leiden, The Netherlands

Introduction:

Evaluating the kinetics of the different functional CD4+ T-cell subsets is essential for immune monitoring of novel anti-tumor or anti-infectious immunotherapy, among other immune-related disease conditions. However, standardized multiparametric flow cytometry (MFC) panels for this goal are still lacking in routine clinical settings. Here we report on the design and validation process of a single 14-color combination of monoclonal antibodies (Mab), aiming at the identification of the most relevant functional CD4+ T-cell subsets in different maturation stages.

Methods:

A total of 73 Mab were evaluated in successive rounds of testing and designing, for the selection of CD4+T-cell markers on 79 (peripheral blood) PB samples from healthy donors. EuroFlow SOP were used for staining and data acquisition on a Fortessa X20 flow-cytometer. To verify that the selected markers did identify specific functional CD4+T-cell subsets, two validation approaches were carried out: i) GEP (gene expression profile) assay of 85 genes –performed in 22 sorted CD4+ T-cell subsets/case from 6 healthy donors-; and ii) intracellular cytokine-production assay after *in vitro* stimulation with PMA+Io and CMV lysate (n=2). The comparability and reproducibility of the results were checked by three reference centers in 16 PB samples.

Results:

During panel design, CD3, CD45 and CD4 were included as mandatory markers for the specific identification of CD4+T cells; CD27, CD45RA and CD62L were selected (from a total of 8 maturation molecules) for the identification of precise maturation stages, due to their high discrimination power, based on principal component analysis. In addition, appropriate Mab were selected, to identify classical, follicular, regulatory and T helper subsets, which could be further divided into near 100 different functional (i.e. Th1, Th2, Th17, Th1-Th17 and Th22, based on their chemokine expression pattern) and maturation CD4+T-cell subsets. Furthermore, cytCD154 was included as a T-cell activation marker. Both GEP and *in vitro* stimulation assays confirmed the association between the phenotypic profiles used for the identification of each cell subset and their actual functional roles. Multicentric validation showed a high reproducibility in CD4+ T-cell subsetting among the three reference centers.

Conclusion(s):

Our results show that the EuroFlow-PERISCOPE 14-color CD4+T-cell tube allows the identification of the main functional and maturational CD4+ T-cell subsets based on their immunophenotype. This is a simple, reproducible and clinically-applicable panel for monitoring CD4+ T-cells in human PB, that may be used in a wide range of clinical settings.

Impact of Flow Cytometry in the Development of a Cellular Immunotherapy with Thymus-derived Treg to prevent rejection in Heart-transplanted Children

Esther Bernaldo de Quirós[1], Rocio López[1], Maribel Clemente[2], Manuela Camino[3], Nuria Gil[3], Esther Panadero[3], Juan Miguel Gil-Jaurena[4], Mª Eugenia Fernández-Santos[5], Marjorie Pion1, Rafael Correa-Rocha[1]

[1]Laboratory of Immune-regulation, Gregorio Marañón Health Research Institute (IISGM), Madrid, Spain, [2]Cell Culture Unit, Gregorio Marañón Health Research Institute (IISGM), Madrid, Spain, [3]Pediatric Cardiology Unit, Gregorio Marañón Hospital (HGUGM), Madrid, Spain, [4]Pediatric Cardiac Surgery Unit, Gregorio Marañón Hospital (HGUGM), Madrid, Spain, [5]Good Manufacturing Practice (GMP) Cell Therapy Unit, Gregorio Marañón Health Research Institute (IISGM), Madrid, Spain

Introduction:

Immune allograft rejection remains the main obstacle to successful transplants. Although adoptive transfer of regulatory T cells (Treg) has acquired growing interest in achieving indefinite graft survival, Treg isolated from peripheral blood showed several limitations. Therefore, we explored the use of thymic tissue as an alternative source of Treg to obtain the maximal yield of cells with the optimal phenotype for use as cellular immunotherapy in heart transplanted children.

Methods:

Thymuses, which are routinely discarded during pediatric cardiac surgery, were collected at the *Pediatric Cardiac Surgery Department* and processed in the Good Manufacturing Practice (GMP) *Cell Therapy Unit* using the CliniMACS (Miltenyi Biotec), after obtaining the inform consent of the patients. Taking advantage of the multi-parameter flow cytometry technology (Gallios, Beckman Coulter), we established several cell quality tests regarding viability, purity, phenotype, functionality and stability, to validate the optimal protocol for the manufacturing of thymus-derived Treg (thyTreg).

Results:

We developed a novel GMP-compatible protocol to obtain autologous Treg from discarded thymuses. The thyTreg product obtained with our protocol showed very high purity (>90% of CD25+Foxp3+ cells), viability of >90%, stability and very high suppressive capacity, inhibiting the proliferation of effector CD4+ and CD8+ T cells in more than 80% (Figure 1). Multiparameter flow cytometry employing 12 parameter analysis was crucial to analyze the phenotype and functionality of thyTreg and to improve the protocol to obtain a very high-quality product valuable for cell therapy in humans.

Importantly, the number of thyTreg obtained from one single thymus reached values of more than $13x10^9$ cells, enough to prepare more than 500 doses of thyTreg. After receiving the approval from the Spanish Drug Agency (AEMPS), we will initiate the first clinical trial worldwide

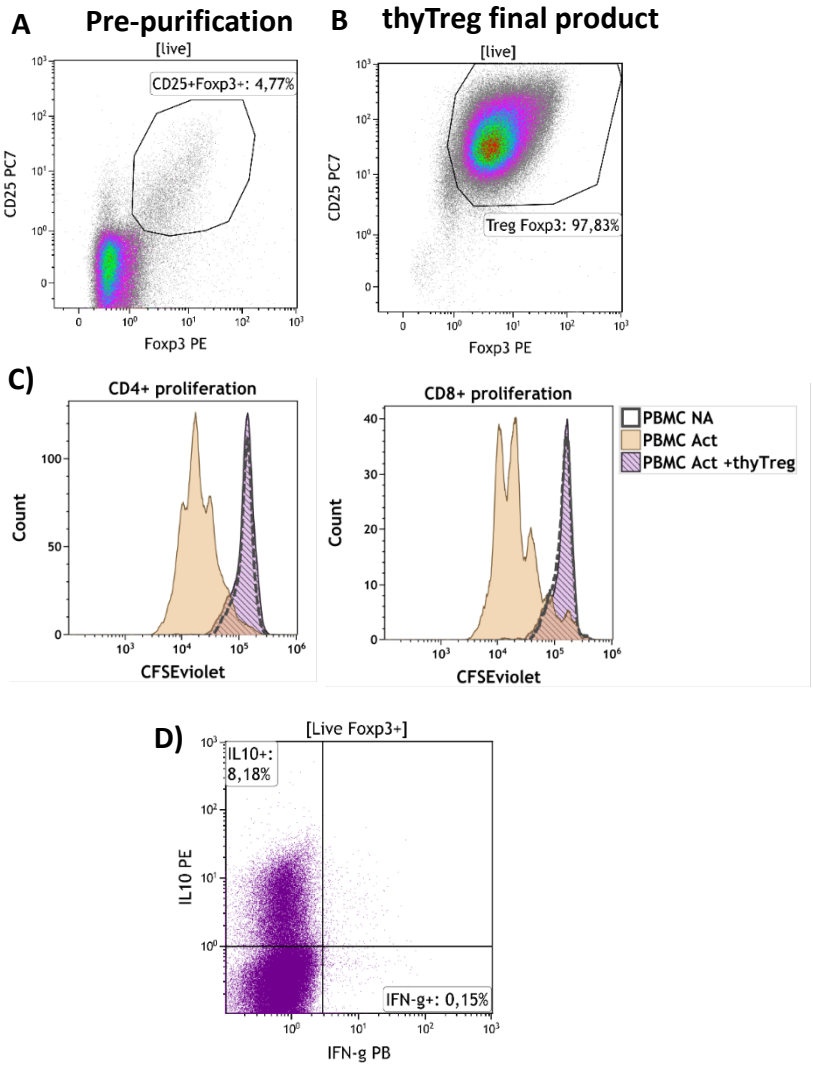
(phase I/II) to evaluate the safety and feasibility of employing autologous thyTreg in children undergoing heart transplantation to prevent the graft rejection by inducing Treg-mediated immune tolerance.

Conclusions:

Massive amounts of pure, stable, highly suppressive thyTreg obtained with our novel GMP-compatible protocol are suitable for use as cellular immunotherapy to prevent rejection in heart-transplanted children. The clinical use of these thyTreg could increase the graft survival in transplanted patients and may revolutionize the treatment of other immunological diseases such as autoimmune disorders.

This work was supported by grants from Fundación Familia Alonso, ISCIII (PI18/00011; DTS18/00038), and CAM (B2017/BMD-3727), co-financed by FEDER funds.

Figure 1: Purity **(A, B)**, suppressive capacity **(C)** and IL-10 secretion of GMP thymus-derived Treg **(D)**.



T-cell subsets and Cytokine profile in Peripheral Blood and Aqueous Humor of patients with Infectious Uveitis

Marta Guedes[1], Maria Arroz[2], Catarina Martins[3], Miguel Dias[3], Rui Proença[4], Luís Miguel Borrego[3,5]

[1]Hospital Egas Moniz, CHLO, Lisbon, Portugal, [2]Hospital S. Francisco Xavier, CHLO, Lisbon, Portugal, [3]NOVA Medical School|Faculdade de Ciências Médicas, Lisbon, Portugal, [4]Centro Hospitalar de Coimbra, Coimbra, Portugal, [5]Hospital CUF Descobertas, Lisboa, Portugal

Introduction:

Uveitis is an intraocular inflammation with either an infectious or non-infectious etiology. It is a potentially blinding disease when left without treatment. The characterization of local and systemic immune profiles in infectious uveitis (IU) could help recognize different clinical entities and contribute to a more targeted treatment approach. In this study, we aimed to characterize T-cell subsets and cytokine profiles in the peripheral blood and Aqueous humor (AqH) of patients with IU and compare them with controls.

Methods:

Eight patients with IU (toxoplasmic, n=2; herpetic, n=6) and 15 controls were included. Controls were cataract patients with no previous history of intraocular inflammation. T-cell subsets in peripheral blood were evaluated by multiparametric flow cytometry using anti-CD3, anti-CD4, anti-CD45, anti-CD45RA, anti-CD197, anti-CD25, anti-CD127 and anti-CD39. Analysis were performed using Infinicyt 2.0^{TM}. A multiplex bead-based immunoassay was used to determine TNF-α, IFN-ɣ, IL-17A, IL-10 and TGF-β levels in serum and AqH samples (BD CBA Flex set, analyzed with FCAP Array software). All acquisitions were performed in BD-FACSCantoII^{TM} flow cytometers.

Results:

Circulating naive $CD3^+CD4^-$ T-cell counts (predominantly naïve CD8 T-cells) were lower in IU patients when compared to controls (p=0,007). Patients also showed lower percentages of naïve Tregs ($CD4^+CD25^{hi}CD127^{low}CD45RA^+CD197^+$; p=0,043) and higher percentages of memory Tregs ($CD4^+CD25^{hi}CD127^{low}CD45RA^-CD197^{+/-}$; p=0,045). Two patients with herpetic anterior uveitis had additional cellular AqH analysis and showed decreased CD4/CD8 ratios (1,13 and 0,66). As for serum cytokine profiles, patients presented higher IL-10 levels when compared to controls (p=0,038). In AqH samples, patients (n=5) showed increased concentrations of IL-10 (p=0,007), TNF-α (p=0,035) and IFN-ɣ (p=0,026) compared to controls (n=8). Interestingly, controls presented higher levels of IL-10, IFN-ɣ and TGF-β in serum samples compared to those found in AqH (p<0,001, for all cytokines). In patients, however, only TGF-β showed higher serum concentrations compared to AqH (p=0,004).

Conclusions:

IU cases showed lower circulating naïve CD8 T-cells counts with a decreased CD4/CD8 ratio in the AqH samples of two patients, possibly denoting an enhanced differentiation pattern in this compartment, related to a viral etiology. Moreover, the imbalance found between naïve and memory Tregs observed in IU patients may be responsible for the augmented IL-10 concentration found in both peripheral blood and local samples. Finally, the elevation of intraocular IL-10, TNF-α and IFN-ɣ observed in IU patients underlines the potential application of these biomarkers in establishing this diagnosis, especially when a viral etiology is suspected. Future studies will allow the confirmation of these applications.

BIOTECHNOLOGY AND FUNCTIONAL CYTOMETRY 1

Flow cytometry quantification of granulocytic alkaline phosphatase activity in unlysed whole blood

Jorge Bardina[1], Laura Garcia[1], Jordi Juncà[1], Àngel Bistué-Rovira[1], Michael Ward[2], Jolene Bradford[2], Jordi Petriz[1]

[1]Josep Carreras Leukaemia Research Institute (IJC), ICO-Hospital Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona (Barcelona), Spain, [2]ThermoFisher Scientific, Eugene, USA

Introduction:

The determination of Granulocyte Alkaline Phosphatase (GAP) activity is used as a simple and useful test in several hematological diseases and malignancies, being the most common assessment method used in modern medicine. Based on microscopy visualization of a cell-bound brown precipitate, 100 neutrophils are assessed on a blood smear, and categorized into 3 to 5 different color-intensity categories. Since this method is observer-dependent, we have developed a new flow cytometry no-lyse no-wash application, using a fluorogenic live cell permeant substrate for alkaline phosphatase.

Methods:

EDTA anticoagulated peripheral blood samples from 29 blood donors were used for this study. Leukocytes were stained with Vybrant DyeCycle Violet Stain (ThermoFisher) following manufacturer's instructions. Samples were incubated with APLS at 37ºC. APLS controls were prepared with incubation at 4ºC to set the autofluorescence by decreasing the enzyme activity. Samples were analyzed on the Attune Acoustic Focusing Cytometer (Thermo Fisher). APLS fluorescence intensities were classified as Level 0 (L0) for non-APLS activity and Level 1 (L1) to 4 (L4) for each upper decade of fluorescence intensity based on the increase of the different levels of the APLS activity. The formula used to calculate the APLS index was the following:

APLS index = (((Median_L1 – Median_L0) / (rSD_L0 * 2)) * (%_events_L1)) + (…) + (((Median_L4 – Median_L0) / (rSD_L0 * 2)) * (%_events_L4))

Results:

Comparisons between cytochemical and FC methods showed no significant differences in terms of index scores (One-way ANOVA) neither when pairwise comparison were used (CI 95%, P-value = 0.085). Two samples exceeded -1.96 rSD (+274.8; -359.5) in Bland-Altman concordance analysis test.

Conclusions:

The staining and analytical methods reported here facilitate the detection, and quantification of subpopulations of leukocyte cells that express alkaline phosphatase activity. These experiments demonstrate the value of flow cytometry as an adjunct to conventional cytochemical methods to quantify the granulocytic alkaline phosphatase activity in unlysed whole blood.

Role of Functional Cytomics in Cancer Immunotherapy and Leukaemia Research

Laura Garcia[1], Jordi Juncà[1], Jorge Bardina[1], Àngel Bistué-Rovira[1], Michael Ward[2], Jolene Bradford[2], Jordi Petriz[1]

[1]Josep Carreras Leukaemia Research Institute (IJC), ICO-Hospital Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona (Barcelona), Spain, [2]ThermoFisher Scientific, Eugene, USA

Introduction:

Flow cytometry immunophenotyping has become one of the mainstream applications for the diagnosis and classification of hematologic neoplasms. This technology is necessary for detection of leukemic blasts and aberrant expression of antigens, and clonal lineage assignment, and is extremely importance in treatment decisions.

However, immunophenotyping does not always provide complete information about the disease, since leukemic stem cell (LSC) phenotype remains unknown or antigen expression may depend on different pathological processes. To overcome these limitations, functional flow cytometry testing can effectively provide new insights for research and evaluation of the disease, and a more complete understanding of the complexities and challenges in the analysis of LSCs and antigen expression.

Here we provide two examples of functional flow cytometrybased research applied to the study of LSC and antigen detection for cancer immunotherapy that could be used in clinical practice. The first one analyzes the alkaline phosphatase (ALP) activity in leukemic blasts in order to detect primitive LSC. The second one is aimed to detect PD-L1 expression at single cell level with a stimulatory functional assay to develop a screening test to determine success of immunotherapy targeting this antigen.

Methods:

Human bone marrow samples from patients diagnosed with acute myeloid leukaemia (n=43) and multiple myeloma (n=31) were collected. For functional analysis, no lyse no wash (NLNW) procedures were applied using Vybrant DyeCycle Violet to discriminate nucleated cells from erythrocytes and debris. Samples were acquired using the Attune™ NxT Flow Cytometer (ThermoFisher).

Results:

In the leukemic stem cell detection assay, ALP activity levels were analyzed in leukemic blasts from several acute myeloid leukaemias, showing a subpopulation with high ALP activity associated with poor prognosis. >12% of ALP+ blasts comprised a patient population with a significant worse prognosis. NLNW methods provided a more accurate detection of ALP activity compared with ammonium chloride and paraformaldehyde lysis-based methods. In the PD-L1 screening assay, we investigated the changes in PD-L1 expression after PMA treatment and found

negative to positive conversion, from 1 to 418-fold increase after stimulation. Moreover, the combination with the immunotherapy showed different responses among patients.

Conclusions:

Functional cytomics can provide new insights for research and evaluation of hematological diseases in the field of LSC detection, associated with cancer resistance, and in the development of screening assays to determine whether cancer immunotherapy can be effective. NLNW procedures provide minimal sample preparation and manipulation to study live cell function and stimuli response more accurately.

Flow Cytometry Osmotic Fragility Test for Screening of Hereditary Spherocytosis. Preliminary data from a reference center in erythropathies

Paula Arana[1], Marta Alonso[1], Marta Riñón[1], Rafael Del Orbe[1], Beatriz Arrizabalaga[1], Natalia Maruri[1]
[1]*Hospital Universitario de Cruces, Barakaldo, Spain*

Introduction:

Among red cell membrane disorders, hereditary spherocytosis (HS) is one of the most common causes of inherited hemolytic anemia. Red blood cell (RBC) morphology, traditional osmotic fragility test and eosin-5'-maleimide binding tests are currently the standard laboratory tests for HS screening. Moreover, flow cytometry osmotic fragility (FCM-OF) test has been reported to be a useful diagnostic test for HS. In this study, we correlated results of FCM-OF with other HS diagnostic tests and showed that FCM-OF could successfully diagnose HS.

Methods:

Blood samples of a total of 8 cases suspicious of RBC membrane disorders were analyzed through FCM-OF and diagnoses were made based on RBC morphology, traditional osmotic fragility test, eosin 5´-maleimide binding test, ektacytometry, analytical data and HS related-mutation screening by next generation sequencing. A total of 48 healthy individuals were also recruited to perform FCM-FO test. Briefly, a red blood cell suspension was spiked with deionized water, a hemolysis-inducing agent, during acquisition and the count of residual red cells was measured sequentially in real-time using flow cytometry. The degree of osmotic fragility was expressed as the "percentage of residual RBCs (%RBC)".

Results:

A reference range (mean ± 1.96*standard deviation) was stablished with %RBC data obtained from healthy individuals. All patients with definite diagnosis of HS (n=5) showed decreased %RBC compared to reference range. Moreover, in 2 of the 5 HS cases eosin 5´-maleimide binding test was not able to detect HS while FCM-OF test did (Table 1).

Conclusion:

FCM-OF test is a simple, quantitative, objective, and cost-effective tool for the measurement of osmotic fragility. In accordance with previous studies, our preliminary data suggest this test as an effective approach for HS screening, although further data is required in order to obtain more robust results.

TABLE 1

Case #	Definite diagnosis	Mutation	FCM-OF, residual RBC % *(reference range)*	EMA binding test	ECM	RBC morphology
1	HS type 3	SPTA1	Low. 32,8%. *(43,3-76,3%)*	Negative	NP	Spherocytes observed. Anisocytosis.
2	HS	ANKIRIN	Low. 34,8%. *(43,5-76,1%)*	Positive	NP	Frequent spherocytes (3-5%)
3	HS	NP	Low. 17,8% *(43,5-76,1%)*	Negative	NP	Frequent spherocytes (3-5%)
4	HS	IP	Low. 32,6% *(44-75,4%)*	Positive	NP	Presence of spherocytes. Frequent microspherocytes (<10%)
5	HS	NP	Low. 32,2% *(44,4-75,5%)*	Positive	NP	Howell-Jolly bodies. Regular acantocytes (2-3%) Spherocytes (1-2%)
6	AB0 imcompatibility hemolytic anemia	NP	Normal. 62.6% *(43,5-76,1%)*	Negative	NP	Very few spherocytes.
7	Hyperferritinemia	NP	Normal. 52,1% (44,1-75,7%)	Negative	NP	Sphero-eliptocytes.
8	Suggestive of overhydrated stomatocytosis	IP	Lower limit. 46,2% (44,1-75,8%)	Negative	Sugestive of hydrocytosis	Frequent stomatocytes. Few spherocytes.

FCM-OF: Flow cytometry osmotic fragility. ECM: Ektacytometry. HS: Hereditary spherocytosis. EMA: eosin-5'-maleimide. RBC: Red blood cell. NP: Not performed. IP: In progress.

IMMUNOLOGY 2

Rare PID in adults and the role of flow cytometry immunophenotype in the differential diagnosis with hematological disease

Cristina Serrano[1]
[1]*Fundación Jiménez Díaz, Madrid, Spain*

Introduction:

Humoral PID in adult others than common variable immunodeficiency and IgA deficiency, are usually asymptomatic and remain undiagnosed. Flow cytometry (FC) is a useful method in patients with PID based on evaluating B cell subpopulations and functional tests or on determining for the presence or absence of specific markers. We present one example of the utility of FC for the differential diagnostic of a patient with predominantly antibody deficiency whose manifestations overlapped hematological disease.

Methods:

55-year-old woman with dim monoclonal IgA Lambda paraprotein (480 mg/dl) and a 0.01 Kappa/Lambda ratio in serum. The study of gammapathy included bone marrow (BM) aspirate and peripherally blood with cytology and FC study (CD45, CD19, CD20, CD22, CD10, CD38, CD138, CD56, CD27, IgD, IgM, Kappa, Lambda, CD5, CD23, CD11c, IgG/IgA memory B subclasses and IgG/IgA plasma B subclasses). As a result of the findings of FC, Kappa chain constant region gene was sequenced.

Results:

Markers of differentiation and maturation of B cells in BM were normal, with a proper distribution between naive and B memory cells. In spite of this, there were no expression of kappa light chain in late pre-B cells, mature B cells and plasma cells. Plasma cells accounted less than a 1% of total cellularity in BM and presented a normal immunophenotype (CD138+, CD38+, CD19+, CD45+d, CD20-, CD10-, CD22-, CD56-). Sequencing of constant region of kappa light chain gene detected homozygous C194G mutation ("Kappa-chain-deficiency" according to the classification IUIS).

Conclusions:

Although they may go unnoticed, some congenital disorders of the immune system can be diagnosed in adulthood. FC is very useful in the hematological or immune laboratory as a first approximation of these rare PID. Not all gammopathies of unknown significance comes from an only cellular clon.

Identification of a therapeutic target by flow cytometry and instauration of a novel treatment in a pediatric patient with ichthyosis

Rebeca Kennedy-Batalla[1], Esther Bernaldo-De-Quirós[1], Ángela Martínez-Martín[2], Minia Campos[1,3], Marjorie Pion[1], Rafael Correa-Rocha[1]
[1]Laboratory of Immune-regulation; Instituto de Investigación Sanitaria Gregorio Marañon (IiSGM), Madrid, Spain, [2]Department of Dermatology and Gastroenterology. Hospital Infantil Universitario Niño Jesús, Madrid, Spain, [3]Department of Pediatric Dermatology. Hospital General Universitario Gregorio Marañón, Madrid, Spain

Introduction:

Ichthyosis is a family of severe, rare genetic disorders that affect the natural skin keratinization and that seriously compromise the quality of life of these patients. The existing treatments are symptomatic and only rarely satisfactory. The aim of this study was to provide insight on the immunological profile of these disorders (little known to date) in order to identify immune targets to allow the use existing biological drugs as a potential effective and safe treatment.

Methods:

Taking advantage of the multi-parameter flow cytometry technology (Gallios, Beckman Coulter), we performed an exhaustive immune analysis in healthy control children and a 1-year old girl with SAM Syndrome (a subtype of ichthyosis). This technique allowed us to analyze frequencies and absolute counts of near of 40 different subpopulations of lymphocytes, granulocytes, monocytes and NK cells. Moreover, we examined the percentage of T cells with the ability to produce different cytokines. The study was performed in the Immune-regulation Lab of the IISGM. The patient was recruited from the dermatology section of the Niño Jesús Hospital, Madrid.

Results:

The thorough study of the immune system by flow cytometry allowed us to identify an immune profile characterized by very high levels of both Th17 cells and IL-17A producing CD4+ T cells. We hypothesize that these elevated levels could play a crucial role on the clinical symptoms of this patient. In consequence, the pediatrician decided to start a new biological treatment in order to diminish the inflammation. Secukinumab, an anti-IL17 monoclonal antibody, proven effective in psoriasis (which also shows a high Th17 immune profile) was used. Follow up samples were analyzed and we observed extraordinary reductions in both Th17 and IL-17A secreting CD4+ T cells. Moreover, the patient showed remarkable clinical improvements: reduction of the scaling, itching and discomfort, weight gain, etc.

Conclusions:

The use of flow cytometry allows the identification of the immune profile of patients with ichthyosis which could provide insight of the severity of the inflammation; the potential existing treatments that could help improve their symptoms and, therefore, their quality of life. Secukinumab could be used as treatment for patients with ichthyosis that have an exacerbated Th17 immune response.

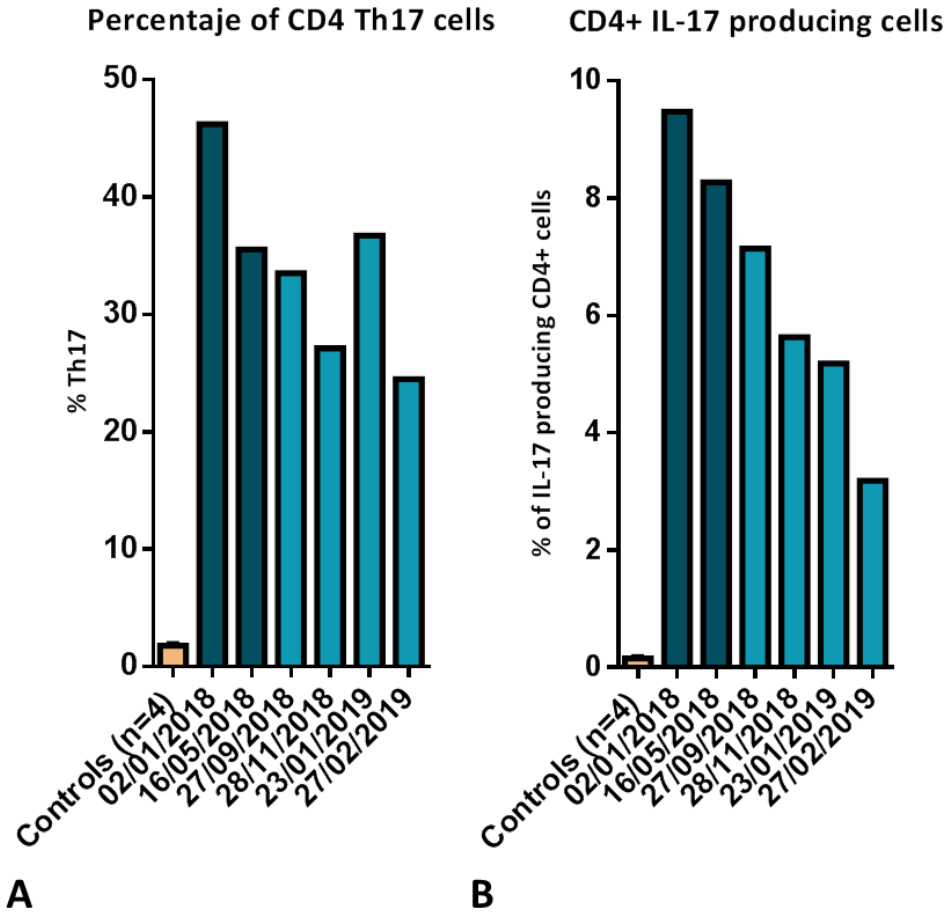


Figure 1:

Follow up samples of patient with SAM Syndrome (female, age = 1). Percentage of **(A)** CD4+ Th7 **(B)** IL-17A producing CD4+ cells.

Beige bars: Mean values of 4 healthy age-matched controls.

Financial support: This work was supported by grants from Fundación Familia Alonso, Instituto de Salud Carlos III (ISCIII) (PI18/00011; DTS18/00038), and Comunidad de Madrid (B2017/BMD-3727) co-financed by FEDER funds.

Complete multilineage CD4 expression defect associated with warts: a phenotypic and genetic study

Rosa Anita Fernandes[2], Martin Perez-Andres[1], Maria Jara-Acevedo[1], Elena Blanco[1], Julia Almeida[1], Alberto Orfao[1], Ines Coutinho[3], Artur Paiva[4], Emilia Faria
[1]Department of Medicine, Cancer Research Centre (IBMCC, USAL-CSIC), Cytometry Service (NUCLEUS), University of Salamanca (USAL), Salamanca, España, [2]Centro Hospitalar e Universitário de Coimbra, Allergy and Clinical Immunology Department, Coimbra, Portugal, [3]Centro Hospitalar e Universitário de Coimbra, Dermatology Department, Coimbra, Portugal, [4]Centro Hospitalar e Universitário de Coimbra, Flow Cytometry Unit- Clinical Pathology Department, Coimbra, Porugal

Introduction:

Although the immunological consequences of CD4 lack of expression has been extensively analyzed in CD4 knockouts (KO) mice, no defects of CD4 gene have been previously reported on humans. Here we present for the first time a case of selective CD4 T-cells lymphopenia due to autosomal recessive mutations in CD4 presenting with extensive treatment-refractory warts in association with undetectable CD4 expression (<0.01 CD4+ cells/uL) affecting multiple cell lineages (e.g. T cells, dendritic cells and monocytes).

Methods and Results:

A 45-year-old female born to consanguineous parents consulted because of exuberant, relapsing and treatment-refractory warts in both her hands and feet, which started at the age of 10 years old, in the absence of other recurrent infections. Histopathological analysis of the lesions confirmed human papilloma virus infection, resistant to different therapies, while the serology and screening for HIV 1/2, HTLV-1, Cytomegalovirus (CMV), *Epstein Barr* virus (EBV) HTLV-1, EBV, CMV and syphilis were negative. Repeated peripheral blood analysis showed absence of CD4+ cells (<0.01%) but normal B-cell NK-cell, and CD8+ T-cell counts. CD4 staining with antibodies directed against 5 different epitopes, located in 2 different domains of the protein, confirmed no cell surface membrane or intracytoplasmic expression on T-cells, as well as on monocytes and plasmacytoid dendritic cells. In contrast, abnormally increased numbers of $CD4^{-}CD8^{-}$ double negative (DN) $CD3^{+}TCR\text{??}^{-}$ T-cells was observed (20% of total lymphocytes; 400 cells/UI), that showed a phenotypic and functional profile similar to that of helper T cells, including the expression of activation/maturation markers, TCRvβ repertoire, and *in vitro* cytokine production profile. The lack of significant alterations in humoral and cytoxic response suggest that DN T-cells are capable to replace traditional helper T-cell lineage. Sequencing of CD4 transcripts revealed two mutations (deletions) affecting the juxtamembrane and juxtamembrane plus transmembrane regions, respectively, resulting in truncated proteins with normal extracellular domains but without anchorage domain to membrane and transmembrane and a premature stop codon.

Conclusion(s):

We report for the first time a case of selective CD4 T-cells lymphopenia due to a CD4 gene defect that translates in complete abrogation of CD4 expression in multiple cell lines, associated with normal cell numbers and recurrent, treatment-refractory HPV+ warts in the patient hands and feet in the absence of other clinical relevant manifestations. Interestingly, a population of DN^- T-cells is expanded that replaces the conventional CD4+ helper T-cells, suggesting that expression of CD4 might be dispensable for helper T-cell lineage selection.

HEMATOLOGY 2

Diagnosis of Therapy-related Acute Myeloid Leukemia with t (8;21) (q22; q22.1) After Treatment for Mantle Cell Lymphoma and Oral Squamous Cell Carcinoma

Maria Cláudia Santos-Silva[1], Patrícia Colombo Corrêa[1], Íris Mattos Santos-Pirath[1], Chandra Chiappin Cardoso[1], Camila Mattiolo[1], Mariana Franzoni Maioral[1], Bruno Vieira Dias[1]
[1]*Universidade Federal De Santa Catarina, Florianópolis, Brazil*

Therapy-related myeloid neoplasms (t-MNs) are a distinct class of malignant neoplasms that occur after cytotoxic chemotherapy and/or radiation therapy. They include therapy-related acute myeloid leukemia (t-AML), myelodisplastic syndromes (t-MDS) and myelodisplastic/myeloproliferative neoplasms (t-MDS/MPN). Despite being rare, t-MNs are highly fatal complications of cytotoxic chemotherapy as they usually carry high-risk karyotypes and have a significantly poorer outcome compared with *de novo* myeloid neoplasms. The incidence of t-MNs is expected to rise in the next years due to the increasing survival rates of cancer patients. t-AML with t (8;21) (q22; q22.1) is rare and has few cases described in the literature[1-4]. In this study we report the diagnosis of a t-AML with t (8;21) (q22; q22.1) that occurred after treatment for mantle cell lymphoma (MCL) and oral squamous cell carcinoma (OSCC).

A 52 years-old male patient diagnosed with MCL was admitted to the University Hospital Professor Polydoro Ernani de São Thiago (HU-UFSC) to start chemotherapy. The morphology analysis of peripheral blood (PB) showed a predominance of small sized cells with high cytoplasm/nucleus ratio and loose nuclear chromatin, whereas some of these cells also presented cleaved nucleus. The immunophenotyping analysis presented 78.1% of B (CD19+) mature (CD20+, CD45++) lymphoid cells, with small size, aberrant strong expression of CD5 and no expression of CD10, CD23 and CD200. Among these cells, 99% presented lambda restriction. The phenotype of these pathological cells was suggestive of MCL with BM involvement, which characterizes MCL in leukemic phase (Figure 1) according to the WHO classification[1]. The treatment consisted in 8 cycles of R-CHOP with rituximab (600mg), cyclophosphamide (1.230 mg), doxorubicin (82 mg), vincristine (1 mg) and prednisolone (20 mg) over 5 months, and the patient experienced remission after treatment.

About three months after lymphoma remission, the patient presented a 1.5 cm tumor in the mucosa of the left molar trigone region. The tumor was biopsied and diagnosed as OSCC, which was surgically removed and treated with cisplatin and radiotherapy. Both these treatments may result in the development of AML or SMD[1].

One month after OSCC treatment, the patient's hemogram showed 35.0% of blast cells and the morphology analysis revealed large sized blasts with basophilic cytoplasm, loose nuclear chromatin and visible nuclei. Besides, some granulocytic cells showed abnormal nuclear segmentation (pseudo-Pelger-Huët nuclei). The PB immunophenotyping showed 38.60% of blasts (CD34++, CD45+), medium to large sized, committed with the myeloid lineage (MPO+, CD13+, CD33+, CD117+, HLA-DR+) and with aberrant expression of CD19 (70%), CD79a (30%) and CD56 (80%) (Figure 2). The aberrant expression of CD19 suggests the presence of t (8;21) (q22; q22.1), which was confirmed by karyotype and Nested RT-PCR.

Blasts of *de novo* AML with t (8;21) (q22; q22.1) and t-AML share morphological, immunophenotypic, cytogenetic and molecular features, although t-AML with t (8;21) (q22; q22.1) seems to have more dysplastic changes than *de novo* AML. These dysplastic characteristics were observed in the patient's granulocytic cells. Based on morphologic, phenotypic and genetic analysis and on the previous historic of chemotherapy and radiotherapy, this case was finally diagnosed as t-AML with t (8;21) (q22; q22.1). The treatment was with (7+3) cytarabine (100 mg/m^2) and daunorubicin (60 mg/m^2).

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Figure 1:

Representative dot plots of pathological cells immunophenotyping analysis revealed cells with low FSC x SSC, expression of CD45, CD19, CD20, CD79a, CD5, lambda restriction CD5 and noexpression of CD10, CD200 and CD23 (red population).

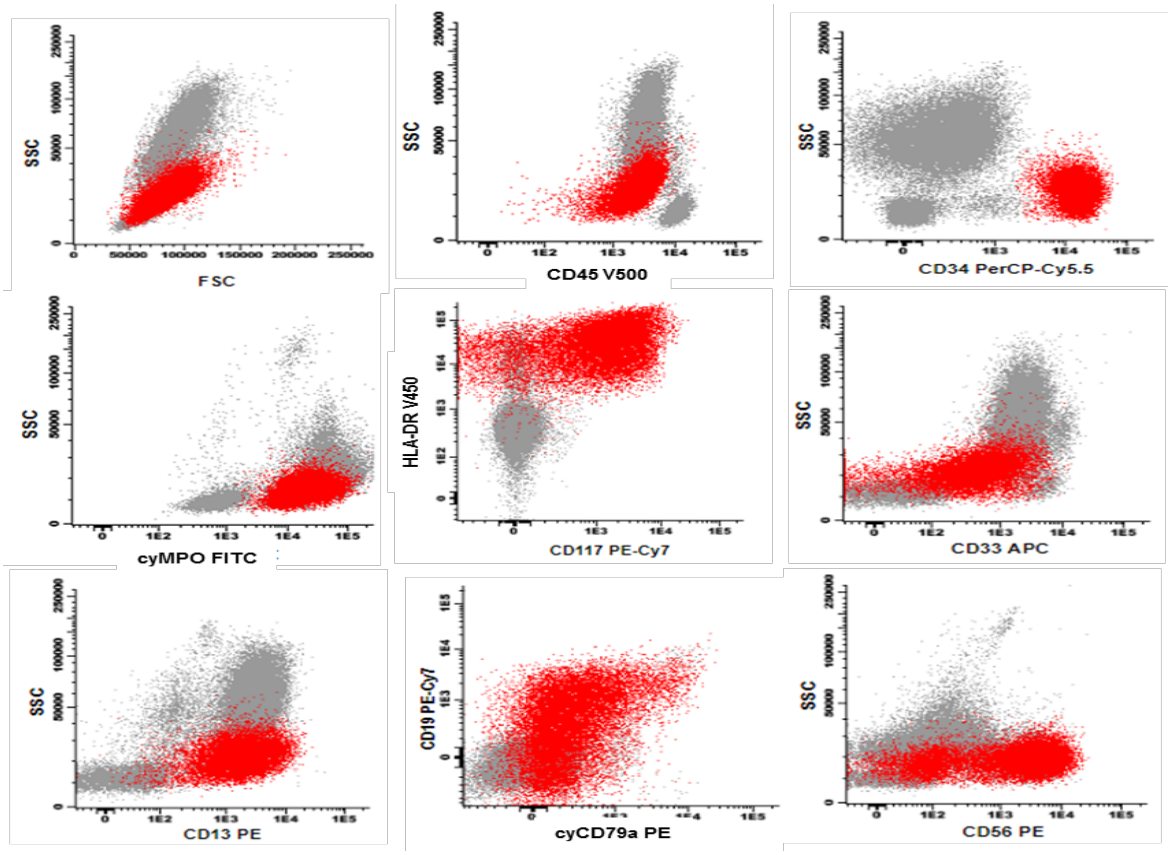

Figure 2:

Representative dot plots showing blasts with high FSC x SSC, expression of CD45, HLA-DR, CD117, MPO, CD34, CD33, CD13 and aberrant expression of CD56, CD19 and CD79a.

Rare nodal gammadelta T cell lymphoma expressing CD4

Christelle Castañon[1], Laura Francisca Ávila Idrovo, Lucia Rita Morais Bras, Covadonga Quirós Caso[1], Enrique Colado[1]
[1]*Hospital Universitario Central De Asturias, Oviedo, Spain*

Clinical case presentation:

An 85-year-old man, without relevant personal history, was referred to the Hematology clinic due to an asymptomatic bilateral cervical enlarged lymph nodes in the last three months. Serological tests for human T-cell lymphotrophic Epstein-Barr virus and hepatitis C virus were negative. No cytopenias were present. Serum lactate dehydrogenase (LDH) was lightly increased 228 U/L (ULN: 225U/L). FNA was performed and fresh unfixed material was sent for immunophenotypic analyses.

Immunophenotypic strategy and results:

We studied FNA material with a multiparametric flow cytometry technique according to EuroFlow cooperative group protocols and a laboratory-developed tube for TCR αβ and γδ testing along TCR delta chain expression (Delta 1 FITC REA173 clone, Delta2 PE clone B6). An expanded TCRγδ T cell population representing 37.8% of viable cells was present. These cells expressed a highly aberrant and infrequent mature post-thymic phenotype: smCD3+ TCR γδ + (with a restricted use of Delta1 TCR chain) TCR αβ - CD4+ CD8-/+d CD56- CD45++ CD43+ CD10- CD19- CD20- CD38+ CD5+ CD7+ CD26- CD2+ CD28+ CD45RA+ CD197+ CD45RO- CD25- cyTCL1- HLADR- CD57- CD30- CD11c- cyPerforine- cyGranzime B- CD16- CD94- CD279-.

Other results:

- Lymph node biopsy: infiltration by large T cells with an aberrant phenotype CD3+ CD5+ CD7+ CD4+ CD8- CD20- CD79a- PAX-5- CD10- Mum-1- BCL-2+ BCL-6- CD30- CD15- CD138- TdT- CD56- GATA- 3+ TCR alfa-beta- TCR gamma-delta+. Ki67 50% of large cells. EBV (LMP-1) was negative.
- Presence of a T lymphocyte population with monoclonal rearrangement of T-cell receptor gene.
- Bone marrow biopsy and peripheral blood: no infiltration.
- PET-CT scan supradiaphragmatic lymph nodes and 2 subcutaneous nodules in scapular regions.

Differential diagnosis:

Reactive populations, T cell lymphoma Not Otherwise Specified (NOS) and T-ALL

Definitive diagnosis:

T cell lymphoma NOS. The most characteristic features in our case of peripheral T-cell lymphoma is simultaneous positivity of lymphoma cells for TCR γδ and CD4. Similar immunophenotypic profile was identified in the larger lymphoid cells and a clonal T cell rearrangement was demonstrated.

These observations suggest a new subtype of γδ T-cell lymphoma, which is characterized by CD4 positivity.

Treatment and evolution:

The patient presented a deep vein thrombosis in the subclavian-axillary region related to lymphoma compression during staging procedures. Treatment with steroids and oral cyclophosphamide, in addition to low molecular weight heparins was initiated, achieving a partial response after two courses.

Discussion:

There are two distinct heterodimer subunits in the T lymphocyte receptor: □□ and γδ. The major subpopulation of γδ T lymphocytes is CD4-CD8- or CD4-CD8+, and manifests cytolytic activity. While nodal γδ T cell lymphomas are infrequent, peripheral CD4+ CD8- γδ T cell lymphoma is an exceptionally rare entity, having only been documented one case in 1996. To the best of our knowledge, no further cases have been documented since then.

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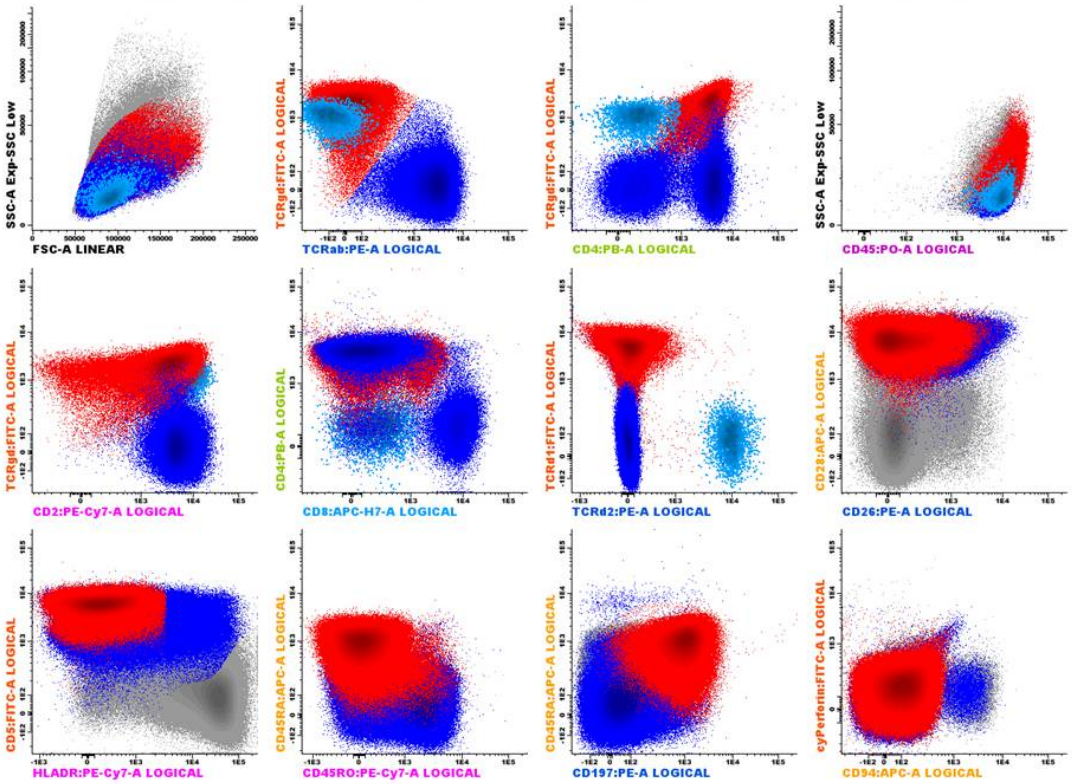

Gated viable events: Blue: Normal αβ T-cells; Cyan: Normal γδ T-cells ;Red:, Aberrant γδ T-cells.

Flow cytometry challenge for the minimal residual disease monitoring in a B-acute lymphoblastic leukemia (B-ALL) patient receiving targeted immunotherapy (TI)

Ana Yeguas Bermejo[1], Alejandro Martín García-Sancho[1], Noemí Puig Morón[1], María Pilar Leoz Allegretti[1], Víctor Higuero Saavedra[1], Irene Aires Mejía[1], Cristina Casquero Hernández[1], Lucía López Corral[1], Estefanía Pérez López[1], Ana África Martín López[1], Félix López Cadenas[1], Alba María Redondo Guijo[1], Álvaro Veiga Vaz[1], Daniel Rivera Delgado[1], Raúl Azibeiro Melchor[1], Daniel Presa Morales[1], Eduardo Sobejano Fuertes[1], Joao Silva Gomes[1], Marta Fonseca Santos[1], Jesús María Hernádez Rivas[1], Marcos González Díaz[1], María Belén Vidriales Vicente[1]

[1]*Hospital Universitario de Salamanca, Salamanca, Spain*

Introduction:

TI with Blinatumomab (Bln) or Inotuzumab ozogamicin (IO) (biespecific anti-CD19/CD3 and anti-CD22 monoclonal antibodies, respectively) are a salvage option for the management of B-ALL with minimal residual disease (MRD), achieving high rates of MRD negative cases, allowing a longer survival or undergo to transplant in a better response situation. However, some patients relapse with a phenotype lacking CD19 or CD22 expression. This resistance mechanism has to be considered in flow cytometry immunophenotyping (FCM) analysis when monitoring MRD.

Clinical case presentation:

A 72-year-old woman consulted in October 2016 for generalized bone pain and severe pancytopenia was observed. Blood smear was normal, but bone marrow aspirate (BMA) was hypocellular with 50% L2-lymphoid blasts.

Immunophenotypic strategy and results:

FCM diagnosis was in a BMA sample, using the EuroFlow BCP-ALL panel. Stained cells were measured in FACSCanto II cytometer equipped with the FACSDiva software and analyzed with the Infinicyt software. We identified 23% blasts with common B-ALL phenotype.

Definitive diagnoses:

Convencional cytogenetic study was normal. Fluorescence in situ hybridization analysis of relevant rearrangements were negative. So, she was diagnosed B-common-ALL negative Ph chromosome.

Treatment and evolution:

She started treatment according to PETHEMA-LAL-OLD-07 protocol. BMA at the end of induction was in complete response (CR) with MRD positive (0.12%). After 3rd consolidation cycle (C), MRD persisted (0.04%), so she started Bln and achieved MRD negative (sensitivity 10^{-4}, S) after 1stC. However, after the 3rdC, a new CD19- blast cell population was detected (0.07%), with a lower expression of CD20, being the remaining markers similar to the diagnostic. In normal B cells (0.05%) the intensity of CD19 expression was conserved. Blast cells increased after 4thC (2.9%) with an associated

morphological relapse (21%). She started IO and reached RC and MRD negative (S 10^{-4}) after 1stC. Treatment was stopped after the 4thC due to upper gastrointestinal bleeding, and in the BMA previous to the next C, CD19- CD20dim CD34+ blast cells were detected again (0.55%). All blasts lost CD22 expression, and only 9% maintained CD10 expression, being the majority CD10 negative. The patient was not candidate to any other therapy. She started palliative treatment and died 2 months later.

Discussion:

TI in patients with persistent or resistant B-ALL disease is increasingly used, improving rates of MRD negative status, allowing them long-term outcomes or to reach a transplant. Although our patient was not a candidate to this approach because of her age, TI was used to prolong her survival.

CD19- and CD22- escape variants have been described under CAR-T cells therapy, and different mechanism of escape have been proposed. Since FCM plays a pivotal role in the monitoring of MRD in ALL, TI raises new challenges and strategies on its detection. In fact, as it is shown in the present case, FCM analysis demands a high degree of expertise and attention to avoid the missing of new populations that could emerge with different phenotype.

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Figure:

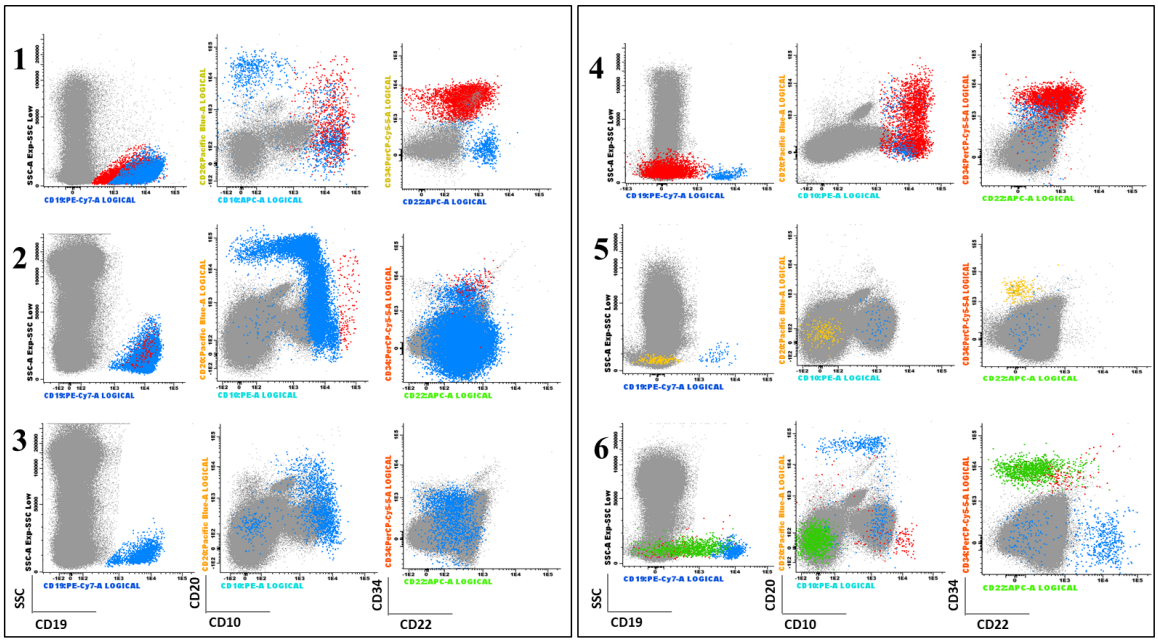

Figure: Illustrating dot plots of FCM analysis at diagnoses and monitoring MRD. **1** Diagnosis (23% blasts); **2** Evaluation before Bln (MRD 0.04%); **3** Evaluation before the 2nd C of Bln (MRD negative, S 10^{-4}); **4** Evaluation after the 4th C of Bln (2.9% MRD); **5** Evaluation after the 1st C of IO (MRD negative, S 10^{-4}); **6** Evaluation before the 5th C of IO (2.9% MRD) . Blue dots: normal B cells; Red dots: blast cells; Green dots: 2º blast cell clone CD10-; Yellow dots: myeloid precursors CD34+ ; Grey dots: residual bone marrow cells. FCM = flow cytometry immunophenotyping , MRD = minimal residual disease , Bln = Blinatumomab, IO = Inotuzumab ozogamicin , C = cycle, S = sensitivity.

BIOTECHNOLOGY AND FUNCTIONAL CYTOMETRY 2

High Throughput Screening of Antibiotic Activity of Gland Uropygial Secretions from House Sparrows by FCM

Alberto Alvarez Barrientos[1], Isabel Clemente Ramos[1], Yolanda Gutiérrez Martín[1], Bettina Perales Casildo[1], Rosa Carrillo del Cacho[1], Sergio Magallanes[2], Alfonso Marzal[2]
[1]Servicio de Técnicas Aplicadas a la Biociencia, Universidad De Extremadura, Badajoz, Spain, [2]Departamento de Anatomía, Biología Celular y Zoología, Universidad de Extremadura, Badajoz, Spain

Introduction:

The uropygial gland is an exocrine gland that produces antimicrobial and antifungal secretions with properties used as a defensive barrier on skin and plumage. This secretion has been proposed to affect the interaction between avian hosts and their ectoparasites (Magallanes et al. 2016). Flow cytometry technique is a rapid, accurate and highly reproducible methodology used in clinical microbiology to monitor antimicrobial activity (Álvarez-Barrientos et al, 2000). It allows to analyze hundreds of samples in a short period of time, in a multiparametric way.

Methods: 222 house sparrows were captured and extracted all the secretion available in the uropygial papilla immediately after capture, following the extraction protocol described by Martín-Vivaldi et al.2009. *Staphylococcus epidermidis* was used to detect antimicrobial action of uropigial secretions that were diluted in DMSO (1:1). Two concentrations and two times were tested (222x2x2=888 samples). *S.epidermidis* was distributed on 96 well plates (200 µL/well) and 1 µL or 2 µL from uropigial secretion diluted in DMSO were added. Control wells consisted in 200 uL of *S. epidemidis* suspension plus 1 or 2 µL of DMSO. After 24h or 48h of incubation plates were centrifuged and 200 uL PI in PBS (0.6ug/mL) was added to each well. Plates were run on a MACSQuant X flow cytometer (Miltenyi Biotec) that allows absolute cell counting (10µL/well). Antimicrobial activity was evaluated by comparison of cell counting (bacterial growth) in wells with presence or absence (controls) of uropygial secretion. Infection by malaria parasite was detected by PCR analysis. Methods were evaluated and approved by Institutional Commission of Bioethics of University of Extremadura (CBUE 49/2011). All the experiments comply with the current laws of Spain, where the experiments were performed.

Results:

A total of 10 plates were analyzed in 2 days (5 plates/day). 96 well plates were analyzed in 35 minutes (3 hours/day). Analysis was performed using Platelogic module from FlowLogic software (Inivai Technologies). Percentage of death cell and absolute counting from each sample were analyzed. Antimicrobial activity of uropygial secretion was significantly higher in uninfected than in infected birds. uropygial gland secretions from female sparrows had a slightly higher antimicrobial activity than secretions from male sparrows, although non-significantly so.

Conclusions:

FCM can be used to study ecological problems in a multiparameter way and with the possibility of analyzing hundreds of samples in a very short period of time.

Flow cytometric characterization of in vitro generation of platelet-derived microparticles in marine mammals

Mar Felipo[1], Alicia Martínez[2], Consuelo Rubio[3], Teresa Álvaro[3], Mónica Valls[3], Carlos Rojo[3], José Enrique O'Connor[1]

[1]Laboratory of Cytomics, Department of Biochemistry and Molecular Biology, University of Valencia, Valencia, Spain, [2]Cytomics Technological Service, Príncipe Felipe Research Center, Valencia, Spain, [3]Comunidad Valenciana Oceanogràfic Foundation, Valencia, Spain

Introduction:

Long-lived cetaceans belong to the top of the aquatic food chain and can thus accumulate environmental contaminants, including many pollutants that have been clearly related to abnormalities of platelet function. A biomarker of platelet activation is the release of plasma-membrane microparticles (PMPs) that contribute to hemostasis regulation through bioactive molecules and signals. However, elevated levels of PMPs are associated with hemostatic disorders, as well as atherosclerosis or angiogenesis processes. The aim of this study is to set up a flow cytometry kinetic assay to characterize PMP formation in vitro and to establish the limit for sensitive detect PMPs after strong platelet activation in whole blood of marine mammals.

Methods:

Citrated whole-blood samples from healthy bottlenose dolphins (Tursiops truncatus), beluga whales (Delphinapterus leucas) and sea lions (Zalophus californianus) were obtained from the Oceanogràfic aquarium (Valencia, Spain). For establishing appropriate gates, whole blood samples were tried with several commercial human antibodies known to identify platelets and leukocytes. A real-time flow cytometric analysis of phosphatidylserine (PS) exposure in platelet surface and PMPs generation triggered by the Ca2+ ionophore A23187 (Sigma-Aldrich) was set up as *in vitro* assay of strong platelet activation. Annexin V-CF Blue (ImmunoStep) was used to determine the rate of PE exposure on platelets, while changes in violet side-scatter (VSS) allowed to detect PMPs formation. The assay was performed in a CytoFLEX flow cytometer (Beckman-Coulter, CA, USA) using VSS as trigger signal to improve the detection of small particles.

Results:

CD41-PE (Clone P2, Beckman Coulter) and CD11a-PECy5 (Clone HI111, Becton Dickinson) were found to label specifically platelets and leukocytes respectively. The real-time cytometric analysis of Annexin V binding to platelet surface and the decrease in platelet forward scatter and VSS intensity demonstrated that A23187 triggered PMPs formation, in a similar way to human platelets in the same experimental conditions. The assay showed different steps in platelet activation and helped to establish the limit for specific detection of Annexin V positive PMPs.

Conclusions:

Specific human CD41 and CD11a clones, and standard Annexin V preparations can be used to identify platelets and leukocytes in dolphins, beluga whales and sea lions. This allows to perform easily whole blood kinetic assayshat may be used as functional biomarkers in toxicological and physiopathological studies. This project has been sponsored in part by a pre-doctoral fellowship from the Conselleria d'Educació, Investigació, Cultura I Esport (Generalitat Valenciana) to M F-B.

Novel Strategy of 16-parameters Flow Cytometry Panel Designing to Analyse Mice Immune System

Elena Blázquez-López[1], Elena Vázquez-Ogando[1], Esther Bernaldo-de-Quirós[2], Rafael Correa-Rocha[2], Rafael Bañares[1], Javier Vaquero[1]
[1]Research Laboratory in Hepatology and Gastroenterology. Gregorio Marañón Health Research Institute (IiSGM), Madrid, España, [2]Laboratory of Immune-regulation. Gregorio Marañón Health Research Institute (IiSGM), Madrid, España

Introduction:

Experimental animal models are an essential tool for investigating the mechanisms as well as diagnostic and therapeutic approaches for many diseases. Among the experimental animals, the mouse is the most used due to its biological similarities to humans and the possibility of genetic manipulations. Although the immunological characterization and monitoring of murine models may provide relevant insights into many disorders (cancer, infections, immunological reactions, hepatic diseases), the small size of these animals represents an important limitation for *in vivo* studies where the amount of blood that can be withdrawn is minimal.

In recent years, flow cytometry has become a powerful technique for providing detailed information on cell populations and their activation state using small volume samples.

The aim of the present study was to develop two panels of antibodies for flow cytometry for carrying out an exhaustive analysis of both myeloid and lymphoid immune cell populations in the mouse using a minor volume of whole blood sample.

Methods:

We designed two multi-parametric panels of antibodies (14 colours) for this project. Whole blood samples obtained by submandibular bleeding or retro-orbital bleeding from C57BL6 mice were collected in tubes containing EDTA. After incubation with the antibodies and erythrocyte lysis, the samples were analysed by flow cytometry using a MACSQuant® Analyzer 16 Flow Cytometer (Miltenyi Biotec, Germany), and the results were analysed using Kaluza Analysis Software (Beckman Coulter, USA).

Results:

Two 14-colour flow cytometry panels were designed according to the requirements of sample restriction, given that the maximum volume that can be collected from mice is 10% of the circulating blood volume i.e. 8 ml/kg or 200 µl for a 25 g mouse.

The two flow cytometry panels allowed the characterization of different immune cell populations including myeloid (monocytes, macrophages, dendritic cells, granulocytes) and lymphoid (T, B, NK and NKT cells) subsets, as well as diverse activation phenotypes and specific polarization of immune responses.

Globally, we were able to analyse more than 35 phenotypes employing between 50 and 100 µl of whole blood per panel.

Conclusion(s):

The reported antibody panels of flow cytometry provide a detailed immunological characterization of the mouse using a reduced amount of whole blood sample, allowing the *in vivo* immunological tracking of mouse models of disease.

Funding:

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POSTERS

1

Improving prognostic significance of the Ogata score in MDS

Joyce R. V. Marques[1], Suiellen Reis-Alves[1], Konradin Metze[1], Irene Lorand-Metze[1]
[1]*University Of Campinas, Campinas, Brazil*

Introduction:

Immunophenotyping of bone marrow (BM) precursors is a useful tool in diagnosis of MDS. Besides, several variables obtained have shown to add prognostic significance to the IPSS-R. The Ogata score has been widely used for discriminating MDS from non-clonal PB cytopenias, but its importance for patients' risk stratification is not well established. Our aim was to analyze the risk stratification power of the Ogata score in consecutive newly diagnosed cases of MDS from our Institution with a long-term observation (median 40 months - 4 to148). We also examined which features could improve its prognostic significance.

Methods:

Diagnosis was made by peripheral blood counts, BM cytology and cytogenetics using WHO 2008 criteria. Deficiency anemias, viral infections and immune diseases had been excluded. Patients' risk was stratified by IPSS-R. Immunophenotyping of BM precursors was made at diagnosis evaluating granulocytic and monocytic maturation and quantifying $CD34^{+}$ cell subsets. In parallel, the Ogata score was assessed (della Porta et al 2012). Patients receiving chemotherapy or BM transplantation were censored at start of treatment.

Results:

77 patients entered the study: 49 men and 28 women. Median age: 67 years (31 – 79); 55 cases were low risk WHO types and 22 cases were RAEB. According to IPSS-R, 10 cases were very low risk, 28 were low risk,19 were intermediary, 16 were high and 4 were very high risk. Ogata score: 0 = 12 cases, 1 = 21 cases, 2 = 24 cases, 3 = 11 cases and 4 = 9 cases. At the end of the observation period, only 17 (22%) patients were alive. In the univariate Cox model, the IPSS-R ($p < 0.00001$) and the Ogata score ($p = 0.001$) had an impact on overall survival, but only the former retained significance in the multivariable model. Among the 4 parameters of the Ogata score, only "$CD34^{high}$" and "$CD34^{low}$" were significant. Besides, myeloid progenitors measured by their phenotype (CD13 and/or CD117) had a higher

impact on prognosis than "CD34^{high}". The same was the case for B lymphoid progenitors (H1). The most important variables adding prognostic significance to IPSS-R were "% CD34 myeloid", "%H1" by phenotypes and "%CD16+ monocytes".

Conclusion:

In the Ogata score, only the parameters quantifying CD34^{+} progenitors have an impact on prognosis. This is improved if progenitors are characterized by phenotype and not by SSC, which is always feasible if appropriate panels are used for immunophenotyping

2

Is Fine Needle Aspiration cytology and multidimensional flow cytometry enough to rule out lymphoma?

Covadonga Quirós Caso[1,2], Lucia Rita Morais[1], Christelle Castañón[1], Hector Torres[1], Luis Fernández, Marco Antonio García Moro[1,2], Rebeca Alonso Arias[1,2], Sara Alonso Álvarez[1,2], Enrique Colado[1,2]
[1]Hospital Universitario Central De Asturias, Oviedo, España, [2]Instituto de Investigación Sanitaria del Principado de Asturias, Oviedo, España

Introduction:

Excisional biopsy (EB) remains as the gold standard for the diagnosis of non-Hodgkin lymphoma (NHL). Fine needle aspiration (FNA) plus ancillary tests such as multidimensional flow cytometry (MFC) immunophenotyping studies are not recommended due to insufficient sensitivity. We questioned whether a standardized FNA plus MFC protocol could avoid false negatives and rule out patients not requiring an EB for the exclusion of NHL.

Methods:

Adults (>18 years old) with enlarged lymph node (LN) and suspicion of NHL were studied prospectively and in parallel in the Pathology and Laboratory Medicine Departments. Patients with a previous diagnosis of hematolymphoid neoplasm were excluded. FNA specimens were collected via direct, ultrasound or endoscopic ultrasound guided puncture and analyzed by two experienced cytopathologists. MFC were performed following EuroFlow Consortium Standard Operative Protocols. Final diagnosis was obtained according to the WHO Classification of Tumours of Haematopoietic and Lymphoid tissues and/or clinical criteria (resolution of LN enlargement and identification of an immunologic or infectious cause). Samples were considered representative providing adequate celularity and viability for both cytologic and MFC studies (arbitrary cutoff of at least 10^5 viable cells and general viability over 30%).

Results:

From January 2015 to December 2018, we identified 195 consecutive samples (from 192 patients) which were negative for NHL and carcinoma infiltration after FNA and MFC analysis. Final diagnosis was: Reactive lymphadenopathy (RL), 184 cases (94.3%); Classical Hodgkin lymphoma (CHL), 10 cases (5.2%); and Diffuse Large B Cell Lymphoma (DLBCL), 1 case (0.5%) (Although in a different lymph node region that the one in which the original FNA was performed). In 22 patients, FNA, MFC and core/excisional biopsy were

simultaneously performed on the same region. CHL was diagnosed in one case, while the remaining 21 cases were diagnosed with RL. Interestingly, in 42 patients, lymphadenopathy persisted or reappeared, prompting the need for EB. In these patients, CHL was the final diagnosis in 9 cases, RL in 32 cases and DLBCL in 1 case.

Conclusion(s):

FNA cytology complemented with standardized MFC can efficiently rule out non-Hodgkin Lymphoma, while classical Hodgkin lymphoma largely remains undetected. Due to false negative cases (Hodgkin Lymphoma), EB is recommended. In selected cases with low probability of HL and adequate FNA plus MFC showing RL, clinical follow up could be discussed, avoiding excisional biopsy.

3

Mantle cell lymphoma presenting as blastoid variant in leukemic phase. Six years´ experience in an academic institution

Covadonga Quirós Caso[1,2], Sara Alonso Álvarez[1,2], Marco Antonio García Moro[1,2], Ariana Fonseca Mourelle[1], Lucia Rita Morais[1], Rebeca Alonso Arias[1,2], Enrique Colado[1,2]

[1]Hospital Universitario Central De Asturias, Oviedo, Spain, [2]Instituto de Investigación Sanitaria del Principado de Asturias, Oviedo, Spain

Introduction:

Mantle cell lymphoma (MCL) often consists of uniform small lymphocytes with a typical immunophenotypic profile and 11q13 translocations, leading to overexpression of cyclin D1, however some patients develop aggressive diseases mimicking an acute leukemia (AL). These blastoid MCL variants may be more common at disease evolution than at diagnosis and may be difficult to identify.

Methods:

EDTA-K 3 anticoagulated peripheral blood (PB) samples were analyzed in the Core hematology Laboratory, where four automated analyzers operate (two Sysmex XN-20 and two Sysmex XN-10) coupled with a digital cell morphology system (Cella Vision). At least 10 6 viable cells were acquired in a FACSCanto II (BDB, San Jose, CA). Instrument setup and sample preparation was performed following EuroFlow Standard Operative Procedures. Conventional cytogenetics (CC) and iFISH were performed according to standard laboratory procedures.

Results:

From January 2012 to December 2018, we identified 5 patients with no previous evidence of a hematolymphoid disorder with a cytologic suspicion of acute leukemia and whose final diagnosis was MCL, blastoid variant according to the WHO Classification of Tumours of Haematopoietic and Lymphoid tissues. Clinical characteristics at presentation are shown in table 1. All patients presented cytopenias and splenomegaly and 3/5 had enlarged lymph nodes. Multidimensional flow cytometric (MFC) studies showed a typical MCL profile (CD19+ CD20+/++ CD5+ CD45d/+ CD200- CD43+ CD23- CD10- CD34-) in 5/5 cases, prompting to iFISH confirmatory study, which demonstrated CCND1/IGH rearrangement in all cases. CC was evaluable in 2 cases, showing a complex karyotype. iFISH pattern was compatible with hyperploidy in 5/5 cases. Despite treatment, these patients had a very poor prognosis. (Median Overall Survival 2,75 months)

Conclusion(s):

Mantle cell lymphoma may occasionally present as a blastoid variant with predominant leukemic manifestations. These cases cannot readily be differentiated from AL on a cytological basis. MFC and cytogenetic studies enable a correct and timely diagnosis for these patients.

	Case 1	Case 2	Case 3	Case 4	Case 5
Sex	Male	Female	Male	Female	Female
Age (years)	63	79	81	91	51
Hemoglobin (g/L)	116	85	117	86	104
Platelets/µL	123,000	56,000	150,000	89,000	255,000
Leukocytes/ µL	710,000	91,490	17,500	6,000	49,190
% Blasts in Perypheral blood	84	21	27	9	43,7
LDH U/L (NR 135-225)	266	739	397	259	2858
Treatment	R-CHOP	R-Bendamustine	R-Clorambucil 1 course, then Ibrutinib	R-Clorambicil	R-DA-EPOCH
Overall survival (months)	2	2	18	3.5	1*

* Under active therapy

4

CD43 and CD49d from the B-cell chronic lymphoproliferative disorders diagnostic panel are useful to detect erythroid dysplasia

Isabel Silva[1], Sandra Silva[1], Patricia Tomé[1], Melissa Oliveira[1], Paula Laranjeira[2], Manuela Fortuna[1], Rui Bártolo[1], Andre Ribeiro[3], Monica Santos[4], Emilia Cortesão[3], Gilberto Marques[5], Helena Vitoria[4], Leticia Ribeiro[3], Artur Paiva[1]

[1]Unidade de Gestão Operacional de Citometria, Serviço de Patologia Clínica, COIMBRA, Portugal, [2]Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculdade de Medicina Universidade de Coimbra, Coimbra, Portugal, [3]Serviço de Hematologia Clínica, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal, [4]Serviço de Hematologia, Centro Hospitalar Viseu-Tondela, Viseu, Portugal, [5]Serviço de Patologia Clínica, Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Introduction:

Despite bone marrow (BM) immunophenotyping by flow cytometry has progressively been recognized as an important tool for the diagnosis of myelodysplastic syndromes (MDS), the sparse knowledge about the normal erythroid maturation and the lack of markers for erythroid characterization is a major shortcoming.

Methods:

Thus, the objective of this work was to analyze the expression pattern of CD43 and CD49d, two markers included in the diagnostic panel for B cell chronic lymphoproliferative disorders, in the CD34 compartment of normal BM, as well as along the normal and dysplastic erythroid maturation process. For this, 13 normal BM aspirates and 18 BM aspirates from MDS patients (2 MDS with single lineage dysplasia, 6 MDS with multilineage dysplasia, 3 MDS with excess blasts-1 and 7 MDS with excess blasts2), were studied by flow cytometry.

Results:

In normal BM, we found a higher expression of CD43 and CD49d among CD34 erythroid precursors, compared to the CD34 cells committed to the remaining hematopoietic cell lineages. CD43 expression progressively decreased along the normal erythroid maturation. In turn, CD49d levels increased in earlier stages and decreased in the last stage of maturation. In MDS, the expression of CD43 and CD49d followed the same pattern, however, a statistically significant decrease in the mean fluorescence intensity values was observed for all maturation stages of erythroid lineage.

Conclusions:

Our results point to the usefulness of CD43 and CD49d markers in the identification of dysplastic phenotypic features in the erythroid lineage, allowing the selection of patients who would benefit from a more extensive BM immunophenotypic study to evaluate the presence of MDS.

5

Multicentre experience on eosinophilia and flow cytometry (FC) related studies

Ana Yeguas[1], Cristina Serrano[2], Beatriz Álvarez[3], Celina Benavente[4], María-Belén Vidriales[1], Cecilia Muñoz-Calleja[6], María del Carmen Castellanos[7], Ana Pérez-Corral[8], Laura Sánchez-Muñoz[9], Raquel Mata[2], María Sopeña[3], Estefanía Bolaños[4], Alejandro Martín[1], Fabiola Barriopedro[5], Tamara Mateu-Albero[6], Vanessa Ortega[7], Javier Anguita[8], Ana Henriques[9], María Sánchez[2], Alberto López[4], Noemí Puig[1], Ruth Martínez[5], Ligia Gabriela Gabrie[6], Raquel Gonzalo[2], María Pilar Leoz[1], Susana Castañón[2], Selene Ledesma[2], Dolores Subirá[5]

[1]Hospital Universitario de Salamanca, Salamanca, Spain, [2]Hospital Fundación Jiménez Díaz, Madrid, Spain, [3]Laboratorio Central de la Comunidad de Madrid BR Salud. Hospital Infanta Sofía, San Sebastián de los Reyes, Spain, [4]Hospital Clínico San Carlos, Madrid, Madrid, [5]Hospital Universitario de Guadalajara, Guadalajara, Spain, [6]Hospital La Princesa, Madrid, Spain, [7]Hospital QuirónSalud, Pozuelo de Alarcón, Spain, [8]Hospital Gregorio Marañón, Madrid, Spain, [9]Instituto de Estudios de Mastocitosis. H. Virgen del Valle. Complejo Hospitalario de Toledo (CLMAST-HVV), Toledo, Spain

Introduction:

A wide range of non-haematopoietic conditions and haematopoietic neoplasms (HN) associates eosinophilia. A FC study might be requested though its diagnostic yield isn´t well-known. This work describes the FC protocols applied in several centres for samples requested for eosinophilia and describes its association with HN.

Methods:

A survey was designed to investigate current practices of clinical FC laboratories when “eosinophilia” was the reason for consulting. Participants reported information on their FC workflow and final clinical diagnosis.

Results:

Between 2016 and 2018, the 9 centres that entered the study received a total of 60,687 immunophenotyping studies. Eosinophilia was the reason for request in 266 samples: 210 (0.34%) peripheral blood, PB; 55 (0.09%) bone marrow, BM and 1 urine. The frequency of this request per year among laboratories ranged between 0.05-1.74% in PB, and 0.07-0.7% in BM. Only 17 patients had serial evaluations. Median eosinophil count (218 cases had available information) was 1,200/mm^3 (range <100-33,563).

After reception, 46 samples (17.3%) were discarded for FC study because eosinophilia wasn´t confirmed. A sequential strategy for staining was used in 8/9 centres. They all included CD3, CD4, CD8 and CD45 in their screening panel, 7 added CD5 and B-cell markers, and 2 centres studied CD7. To characterise abnormal T-cell populations, laboratories

designed extensive and heterogeneous home-made panel, with agreement on 12/36 reagents in >50% laboratories.

A clinical diagnosis was available in 150/220 cases (68.2%), mostly reactive conditions (n=102/150, 68%). In the remaining 48 cases (42 patients), eosinophilia was associated with myeloid malignancies (2 acute leukemias, 2 myelodysplastic syndromes, 5 myeloid neoplasms with rearrangement of PDGFRA/PDGFRB; lymphoid malignancies (5 B-CLL, 2 Hodgkin´s disease, 2 clonal gammopathies, 6 T-NHL, 1 NK malignancy); 2 autoimmune thrombocytopenia, 1 systemic mastocytosis and 1 hyper-IgE syndrome. Hypereosinophilic syndrome was established in 14 samples and lymphocyte-variant hypereosinophilia in 5.

FC detected abnormal T-cell populations in 18 cases: 1 angioimmunoblastic T-NHL, 1 large granular lymphocytic leukemia, 5 lymphocyte-variant hypereosinophilia and 3 reactive conditions. Eight cases lacked a conclusive diagnosis. Median percentage of abnormal T-cells as referred to total lymphocytes was 12.5% (range 0.02-50%).

Conclusions:

Since most eosinophilias were reactive, a FC screening of all lymphoid populations seems reasonable. In our series, lymphomas were infrequent, with a similar FC detection of B-CLL and T-NHL. FC was crucial to diagnose lymphocyte-variant hypereosinophilia. However, in 30% of cases with abnormal T-cells, follow-up and TCR molecular studies seem mandatory to determine its significance.

6

Coexistence of lymphoplasmacytic lymphoma, multiple myeloma and myelodysplastic syndrome

Sara Cáceres Hernández[1], Helena Bañas Llanos[1], Maria José Arcos Carmona[1], Rocío Cardesa Cabrera[1], Sara Suárez-Varela[1], Ignacio Casas Avilés[1], Baldomero Moriano Palacios[1], María Espina García[1], Ángel Rodríguez López[1], Fátima Ibañez Espacio[1], Nuria Bermejo Vega[1], Carmen Cabrera[1], Fernando Carnicero González[1], Juan Bergua Burgués[1]
[1]*Hospital San Pedro de Alcántara, CACERES, España*

Introduction:

Concomitant hematologic malignancies are unusual and difficult to diagnose. The coexistence of lymphoplasmacytic lymphoma (LPL) and multiple myeloma (MM) is infrequent, there are only a few cases reported. Third hemopathy's association is extremely uncommon. We report a case with simultaneous LPL, MM and myelodysplastic syndrome (MDS).

Clinical case:

75 years old man with diabetes´s personal history who was diagnosed of MDS type refractory anemia with ringed sideroblasts (RARS). He was asymptomatic and was followed with periodic control. Three years later he experienced a decrease of haemoglobin's levels, neutropenia and bone pain. Laboratory studies showed now haemoglobin 6.2 g/dl, neutrophils 100/ul, increase of IgG 4284 mg/dl and IgM 700 mg/dl with two monoclonal bands IgG kappa 1.87 and IgM 0.07 g/dl in serum protein electrophoresis.

BM aspirate showed hypercellularity with dysplasia in the three lineages, 15,5% of lymphocytes, 18% of atypical plasma cells and a proliferation of masts cells without blasts. Biopsy result was in agreement.

Karyotypic analysis showed 10% -11q13; Fluorescence in situ hybridization was negative and sequencing study of myeloid differentiation primary response gene 88 (MYD88) was positive for the L265P mutation.

CT was normal and in the radiography study lytic lesions and spinal crushing were observed.

Immunophenotypic study:

BM sample was studied underwent MM assessment with Euroflow protocol.

Flow cytometric analysis of bone marrow demonstrated a lymphocytosis with a monoclonal B cell population kappa CD38 negative and a 5.2% of plasma cells with two different populations both of kappa clonality of different intensity. One of them, with smaller size and positive for CD45hi, CD19 and CD27 and CD56 negative. The other majority population, with larger size and lower expression of CD38, CD45 (weak), CD81 and beta-2-microglobulin positive and negative for CD19, CD117, CD27 and CD28.

He started with rituximab, bortezomib and dexamethasone regimen. He has received 6 cycles with partial response, clinical improvement, transfusional dependency and decrease of the IgM and IgG monoclonal band of 1.2 g/dl. Bone marrow revaluation is pending.

Discussion:

The association of LPL and MM is very infrequent and has been reported in only six patients previously. There are no cases of coexistence of these three hematologic malignancies. An integration of clinical, morphologic, immunophenotypic, molecular and cytogenetic studies to obtain the correct diagnosis is necessary.

There is no specific immunophenotypic or cytogenetic abnormality in LPL. The MYD88 L265P mutation is present in 91% of LPL patients and useful to differentiate LPL of IgM producing myeloma what is negative and of the most of low-grade B cell lymphomas that are also negative.

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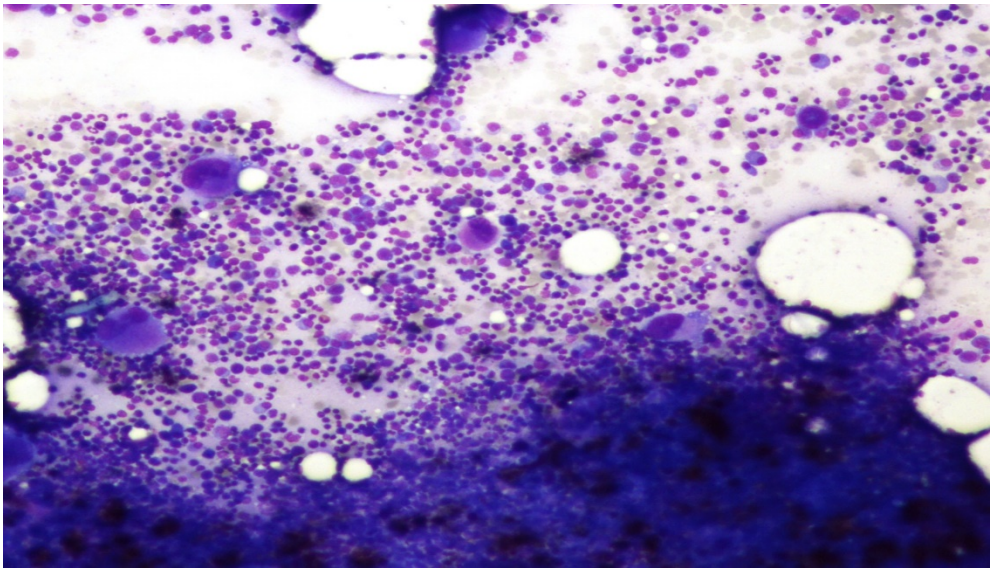

7

Chronic anemia due to Pyruvate Kinase deficiency disrupts the hematopoietic stem cell compartment

Rebeca Sanchez-Dominguez[1], Omaira Alberquilla[1], Sergio Lopez-Manzaneda[2], Sara Fañanas Baquero[2], Oscar Quintana-Bustamante[2], Susana Navarro-Ordoñez[2], Jose Carlos Segovia[1,2]
[1]LACISEP, CIEMAT, Madrid, Spain, [2] Advanced Therapies Unit. CIEMAT, Madrid, Spain

Introduction:

Human pyruvate kinase deficiency (PKD), an autosomal recessive disorder produced by mutations in the PKLR gene, is the most common cause of chronic nonspherocytic hemolytic anemia. This deficiency alters the erythrodifferentiation and gives rise to anemic phenotypes. In other anemic phenotypes it has been shown that the low red blood cell count have an impact on the compartment of more undifferentiated progenitors, yet the stem cell compartment in PKD remains unclear.

Results:

By studying a mouse model of PKD we have seen a defect within the stem cell compartment, identified by the LSK phenotype. PKD bone marrow showed both lower proportion and absolute number of LSK cells per femur. Consequently, with these data in a competitive BM transplantation setting PKD bone marrow showed an engraftment disadvantage. At the clonal level, the PKD LSK pool had less in vitro proliferative potential both in number and size of clones in when compared with WT LSK cells. Within the LSK compartment, we have found a decrease of multipotent progenitors (MPP) in favour of an absolute increase of LSK FLK2-CD34- compared with WT animals, although the proportion of HSC with the CD150 SLAM marker in their surface was lower. Thus, self-renewal and reconstituting potentials of PKD LT-HSC compartment declined as CD150 expression levels decline.

Conclusions:

Overall, our data indicate that the lack of PK enzyme occurring in PKD mice, leads to an imbalance in the most immature hematopoietic progenitor compartment. Whether this observation is due to intrinsic changes to the stem cells themselves and/or to different extrinsic signals received through a hyperstimulated or structurally modified hematopoietic niche by the constant bias towards the erythroid path is under study.

8

Pancytopenia and cutaneous lesions in an elderly patient. Importance of comprehensive diagnosis

Christelle Castañón Fernández[1], Ana Alicia Lopez Iglesias[2], Lucia Rita Morais Bras[1], Covadonga Quirós Caso[1], Ana Díaz Trapiella[2], Ariana Fonseca Mourelle[1], Sara Alonso Álvarez, Marco Antonio García Moro[1], Enrique Colado[1]

[1]Hospital Universitario Central De Asturias, Oviedo, Spain, [2]Hospital del Oriente de Asturias, Arriondas, Spain

Clinical case presentation:

An 84 years old female was referred to the Hematology clinic due to pancytopenia. (Hemoglobin 93g/L; Leukocytes 2.6x10^9/L, Platelets 68x10^9/L) and skin lesions, indurated and violaceous nodules, disseminated over the trunk and scalp. Also, she complained of dyspepsia and weight loss of 13 kg in 6 months. Serum lactate dehydrogenase (838 U/L), uric acid (10 mg/dl) and creatinine (1.35 mg/dl) were elevated. She had a previous history of left mastectomy due to breast neoplasm (8 years ago) with no further therapy, and hiatal hernia.

A cutaneous biopsy was performed, showing atypical myeloid cells and a second sample along with bone marrow aspirate was sent for immunophenotyping.

Immunophenotypic strategy and results:

Immunophenotypical studies were performed strictly according to EuroFlow cooperative group protocols. After mechanical disaggregation, a cellular suspension from a cutaneous nodule was studied, in which aberrant monocytic progenitor cells (representing 94% of viable events) was detected: FSCint/hi SSCint CD34-/+d CD45+ CD117- HLADR+++ CD56+++ cyMPO- cyCD79a- CD19- CD7- cyCD3- smCD3- CD16- CD13+d CD11b- CD10- CD64+++ CD35- CD14-/+d CD300e- CD36+d CD105+d CD33+++ CD71+d nuTdT- CD15-/+d NG2-/+d CD22+ CD38+ CD123+ CD203c- CD42a- CD61- CD25- CD41- CD4+ CD9+ CD41- CD42b-

Regarding the bone marrow sample, 3.6% of viable cells were precursors similar to those found in the skin nodule. Also, 0.2% of more immature aberrant monocytic progenitors were detected, with blocking maturation, possibly a more undifferentiated population.

Differential diagnosis:
Hematotropic myeloid neoplasms with CD56 expression.

Definitive diagnosis:
Cutaneous infiltration is compatible with a leukemia cutis (LC). Immunophenotypic profile suggests a CBFB/MYH11-rearranged AML as first possibility, which was confirmed on iFISH study. Bone marrow blast cell count on morphology was 3% due to collagen fibrosis as shown in bone marrow biopsy. Therefore, AML with inv (16) (p13.1q22) or t (16;16) (p13.1; q22); CBFB-MYH11 was the final diagnosis. Of note, this diagnosis must be assigned irrespective of the blast cell count on bone marrow.

Treatment and evolution:
The patient was started on semi intensive chemotherapy (Fluga). Treatment induced a partial response on skin nodules and suffered an acute myocardial infarction with a torpid evolution and was sent to hospice.

Discussion:
LC usually occurs in the setting of a previously diagnosed systemic hematolymphoid neoplasm. In rare cases, LC is the harbinger of a systemic leukemic process. In this case, MFC initially confirmed skin infiltration and oriented to the most probable cytogenetic abnormalities allowing a fast and efficient comprehensive diagnosis. CD56 has constantly been shown to be more frequent in extramedullary leukemia cases.

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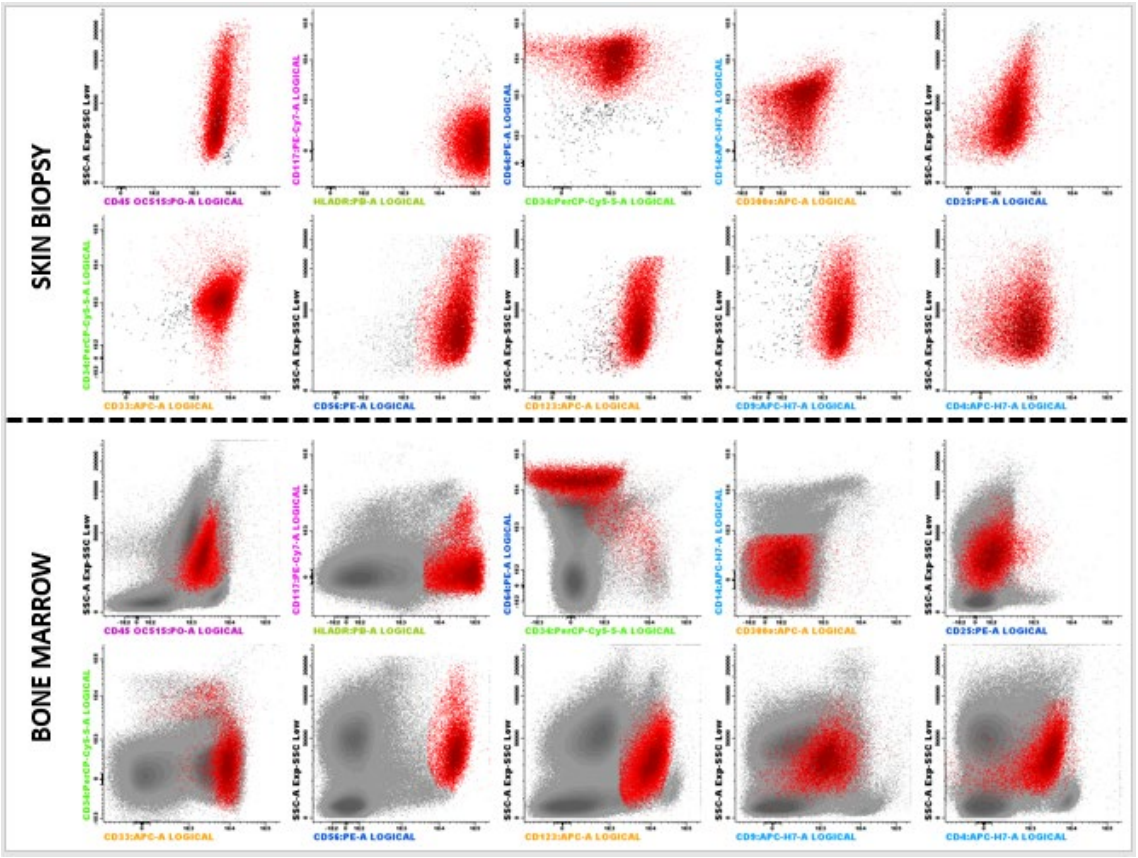
SKIN BIOPSY
BONE MARROW
SSC-A Exp-SSC Low
CD45 OC515:PO-A LOGICAL
CD117:PE-Cy7-A LOGICAL
HLADR:PB-A LOGICAL
CD64:PE-A LOGICAL
CD34:PerCP-Cy5-5-A LOGICAL
CD14:APC-H7-A LOGICAL
CD300c:APC-A LOGICAL
CD25:PE-A LOGICAL
CD33:APC-A LOGICAL
CD56:PE-A LOGICAL
CD123:APC-A LOGICAL
CD9:APC-H7-A LOGICAL
CD4:APC-H7-A LOGICAL

9

New process for multiple myeloma-MRD detection; enhanced sensitivity to 10E-6, 12 color and multidimensional algorithms applied

Josefa Esperanza Marco[1], Mª José Fernández[1], Paz Ribas[1], Amparo López[1], Mar Gonzalez[1], Elena Gómez[1], David Ivars[1], Mª José Sayas[1], Javier De la Rubia[1]
[1]*Servicio de Hematología. Hospital Universitario Dr. Peset, Valencia, Spain*

Introduction:

Flow cytometry (FCM) has become a highly valuable method to monitor minimal residual disease (MRD) in multiple myeloma (MM). The depth of complete response is liked to "time to progression" and "overall survival", where MRD negative patients have a better outcome. Current high-sensitive FCM for MRD detection in MM is optimized for 2 tubes 8-color antibody panel with a 10^{-5} sensitivity achieved. Because MRD detection directly depends on sensitivity reached, we explored the possibility to overcome the technical and informatic limitations to develop a new process based in 1 single tube with 12 colors, enhancing sensitivity by 1-log (10^{-6}). Furthermore, we reviewed and compared the last methods based on multi-dimensional reduction techniques to overcome manual analysis issues due to 12 colors.

Methods:

Bone marrow MM samples were processed using an intra-cyto staining with Fix & Perm kit (ThermoFisher). Panel combination was as follow; cyto-Kappa^{FITC}/cyto-Lambda^{PE}/CD19^{ECD}/CD117$^{PC5.5}$/CD200^{PC7}/CD138^{APC}/CD81$^{A.A700}$/CD56$^{A.A75}$/CD38^{PB}/CD45^{KrO}/CD27^{BV650}/ CD28^{BV780}. Data acquisition was performed in 13 colors (5B-3R-5V) Cytoflex (Beckman coulter). Multiparametric analysis have been carried out using Rplugin application in Kaluza analysis software (Beckman Coulter). Script design for this application was developed in Rstudio software in an "R language" environment.

Results:

It has been demonstrated that in MM the coefficient of variation is around 10% regardless of the size of the cluster, even when as few as 20 malignant events are detected, since myelomatous plasma cells (PCs) usually occupy a space in which background events are scarce. So, 20 million of leukocytes is enough to reach a 10^{-6} sensitivity. Because we use 1 instead of 2 tubes, we could stain 3 times the same combination to acquire more than 20 million leucocytes per sample, merging files finally. To avoid computing time issues because high number of events we performed the complete analysis in a CD138+ gated exported

file, keeping all leukocytes in a different file. We studied different multiparametric algorithms (**Figure 1**). Using FlowSOM we could easily identify clonal PCs in few steps combining both manual and unsupervised analysis.

Conclusion:

We have designed a new highly sensitive protocol for MM-MRD detection that let us to overcome the current sensitivity limitation from 10^{-5} to 10^{-6}. The use of 12-color cytometry allows the whole study to be carried out in a single tube, simplifying the technique. We have identified FlowSOM as a new powerful tool to enhance FCM analysis quality.

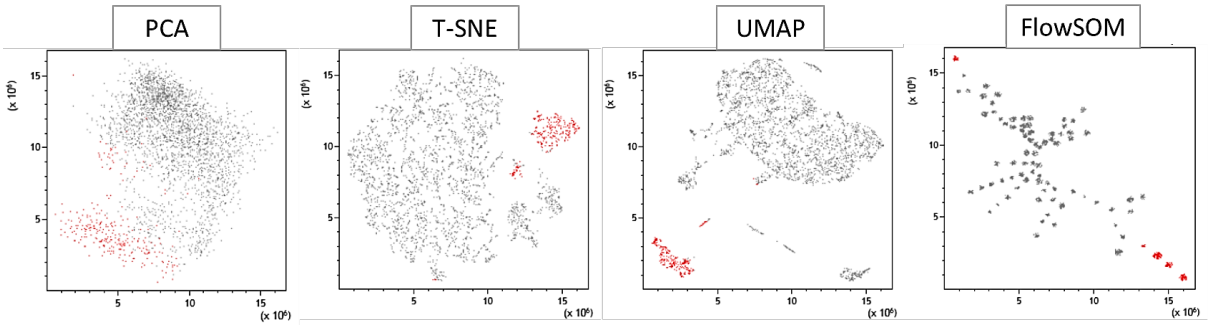

Figure 1. Multi-dimensional algorithms applied to CD138+ gated cells. Red events identify pathological PCs.

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The utility of Flow Cytometry for Detection of Tumor Cells in Cerebrospinal Fluid of Pediatric Patients with Acute Leukemia

Ximena Torres[1,2], Niyireth Peñaloza[1], Iliana de los Reyes[3], Martha Vizcaíno[3], Paula Carolina Guzmán[3], Alba Campos[1], Ana María Uribe[4], Carlos Saavedra[5], Martha Romero[5], Gina Cuellar[5], Liliana Martín[5], Paula Rodriguez[5], Claudia Cardozo[1], Sandra Quijano[1,2,5]

[1]Servicio de Citometría de Flujo, Laboratorio Clínico, Hospital Universitario San Ignacio, Bogotá, Colombia, [2]Grupo de Inmunobiología y Biología Celular, Departamento de Microbiología, Facultad de Ciencias,Pontificia Universidad Javeriana, Bogotá, Colombia, [3]Departamento de Pediatría, Hospital Universitario San Ignacio, Bogotá, Colombia, [4]Departamento de Patología, Hospital Universitario San Ignacio, Bogotá, Colombia, [5]Departamento de Patología. Fundación Santa Fe de Bogotá, Bogotá, Colombia

Introduction:

Infiltration of Central Nervous System (CNS) by acute leukemia (AL) cells is associated with poor prognosis and higher tumor aggressiveness. At present, conventional cytology (CC) is the gold standard approach for detection of leukemic cells in Cerebrospinal Fluid (CSF). This technique has high specificity but limited sensitivity; according to literature, it can reach 20-60% of false-negative results. Under this context, several studies have shown the utility of Flow Cytometry (FCM) for identification and quantification of tumor cells in CSF, because of its higher sensitivity in comparison with CC. This study aimed to evaluate the presence of tumor cells in CSF from patients with AL by FCM and CC, as well as its relationship with clinical and biological parameters.

Methods:

Between 2008 and 2016, 156 CSF samples from 55 patients under 18 years with AL assisted at Hospital Universitario San Ignacio and Fundación Santa Fe de Bogotá, were studied. CSF samples were obtained at diagnosis and during the follow-up after-treatment (on average, three samples per patient, depending on the treatment protocol). CFS samples were drawn by lumbar puncture and split into two equal aliquots. CFS samples were analyzed simultaneously by FCM and CC in those institutions and all statistical analyses were performed using SPSS. The agreement between the techniques was made using the kappa index and Chi-square test.

Results:

Results for malignant cells in CFS samples by FCM and CC were as follows, FCM-/CC-: 131/156 (84%); FCM+/CC-: 19/156 (12.2%); FCM+/CC+: 1/156 (0.64%); FCM-/CC suspicious: 1/156 (0.64%); and FCM+/CC suspicious: 4/156 (2.56%). Concordance between FCM and CC was poor (Kappa coefficient < 0.2). Moreover, patients with B-cell acute lymphoblastic

leukemia (B-cell ALL) and FCM+ results showed a lower response to steroid-treatment on day 8, abnormal karyotype findings, high risk group stratification and neurological symptoms. In addition, FCM+ patients have worse overall survival.

Conclusion:

The present study suggests that pediatric patients with AL will benefit with the implementation of FCM for diagnosis of CNS tumor infiltration, even in the absence of neurological symptoms. CSF analysis by FCM can detect minimal numbers of blasts that could be associated with CNS occult disease. Compared to conventional cytology, the current gold standard for CSF analysis, FCM has the advantage of being a more convenient diagnosis tool because it can detect an occult leptomeningeal compromise, improving directly the correct risk classification and the prognosis of the patients.

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Chronic myelomonocytic leukemia and myelodysplastic syndromes present a continuum of phenotypic alterations in bone marrow myeloid precursors and lymphoid populations

Joyce Rico Vido Marques[1], Fernanda Pereira-Cunha[2], Gislaine B O Duarte[2], Irene Lorand-Metze[1]
[1]University Of Campinas, Campinas, Brazil, [2]Laboratory Sollutio - Campinas, Campinas, Brazil

Introduction:

Recently, the phenotypic features of monocytes in chronic myelomonocytic leukemia (CMML) have been described and there are uncertainties about similarities and differences with those found in low risk myelodysplastic syndromes (MDS). On the other hand, similar immunophenotypic features have been found in juvenile myelomonocytic leukemia. In this entity, also important alterations in bone marrow (BM) lymphoid cells have been described. So, our aim was to study the phenotypic characteristics of myelomonocytic precursors, $CD34^+$ progenitors and lymphocyte subsets in CMML and compare them with normal BM and findings in MDS.

Methods:

We examined cases with CMML diagnosed by WHO 2016 criteria entering our laboratory during 2018. BM phenotypic features were compared to cases with MDS with BM myeloblasts <5%. Cases of transitory peripheral cytopenias presenting a normal BM immunophenotyping were used as controls. An 8-color platform according to Euroflow was used, adding a tube especially for examination of lymphocyte subsets. A minimum of 300 000 cells were acquired.

Results:

We examined 11 controls (age 25-79 years), 23 cases of MDS (age 27-88 years), and 17 CMMLs (age 55-90 years). Concerning immunophenotypc features, SSC of granuloytic precursors was <6 in 10 MDS and in 8 LMMCs. At least 1 abnormal antigen expression was observed in 11/23 MDSs and 11/17 CMMLs. Total monocytes were increased in all CMMLs (median 20.7%) but also in 15 cases of MDS (median 7.2%), with similar proportions of classical monocytes (median 94.7% and 93.1% respectively). Myeloid $CD34^+$ progenitors were >2% in 5 cases of CMML but in none of the MDS cases. B-cell progenitors were decreased in both MDS and CMML.

Lymphoid cells were increased in MDS but not in CMML compared to normal BM (median 23.8%, 10.1% and 15.0% respectively), p = 0.001. This was mainly due to increase of T

lymphocytes in MDS but not in CMML ($p = 0.003$) but the ratio CD4/CD8 was similar in all 3 groups. Mature B-cells were markedly decreased compared to controls (median 14.5; 6.5 and 30.7) $p < 0.0005$). NK and NK-T cells were very variable and showed no significant differences among groups.

Conclusion:

Immunophenotyping of BM in CMML discloses several abnormalities similar to MDS, not restricted to monocytes. Changes in lymphoid cells are more pronounced in CMML than in low risk MDS. They may be predictive of treatment response to 5-azacytidine, that is an immunomodulating drug.

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Application of the eosin-5 maleimide binding test in the study of red cell membranopathies and its differential diagnosis

Beatriz Alvarez Flores[1], Maria Sopeña Corvinos[1], Ataulfo González Fernández[1], Beatriz Soto del Pecho[1], María Medrano Élez[1], Javier García de la Fe[1], Raquel Guillén Santos[1], Fernando Cava Valenciano[1]

[1]*Laboratorio Central de la Comunidad de Madrid. BRSalud, San Sebastián De Los Reyes, Spain*

Introduction:

Red blood cell (RBC) membrane disorders are inherited conditions due to gene mutations leading to a wide spectrum of haemolytic disorders because of a decreased RBC deformability and permeability. They are classified as membrane/cytoskeletal proteins defects (Hereditary spherocytosis-HS-, Hereditary elliptocytosis-HE-, Pyropoikilocytosis-HPP- and Ovalocytosis Southeast Asian) or transmembrane transporters (Dehydrated hereditary stomatocytosis-DHS- and Cryohydrocytosis). The flow cytometric eosin-5 maleimide (EMA) binding test is used for HS diagnosis since King *et al* published its application when a decreased EMA expression was found. Since other membranopathies can also present EMA detriment, other aspects such as RBC morphology and hematrimetric parameters should be considered. The aim of this study is to show the differential diagnosis in 4 types of membranopathies with EMA detriment: HS, HE, HPP and DHS.

Methods:

The EMA-binding test was performed as described by King *et al.* with minor modifications in 4 membranopathies. Samples were processed with at least two normal controls, acquired in FACSCanto II cytometer and analyzed with Infinicyt software. For EMA evaluation the mean fluorescence intensity (MFI) of normal controls was calculated. MFI percentage decrease was calculated using the formula: (MFI controls–MFI patient)/MFI controls×100. The ratio MFI patient/MFI control was also calculated. RBC morphology from peripheral blood smear was reviewed by an expert and ADVIA 2120 counter used to measure RBC hematimetric parameters.

Results:

A significant reduced EMA expression was observed in all cases comparing with controls. HPP showed a great elevation of MICRO% and RDW while DHS presented high HIPER%, MACRO% and MCHC. Blood film review showed differences in the 4 cases studied as described in the table 1.

	HPP	HE	DHS	HS
HIPER%	5,6	7,4	**50,3**	7,1
MICRO%	**19,5**	1,8	5	8,6
MACRO%	2,1	2,8	**7,8**	3,8
HIPO%	7,5	5,7	6,9	**12,7**
MCHC (g/dL)	31,20	34,5	**38,1**	33,7
RDW(CV)	**28,50**	17	23,6	22,6
RETIC%	2.58	6,32	32,09	13,67
EMA detriment %	**23.10**	**33,42**	**21,49**	**23,46**
RATIO EMA	0,76	0,67	0,78	0,77
RBC Morfology	anisopoikylocytosis, small elliptocytes microcytic poikilocytes	frequent elliptocytes	excentrocytes, dehydrated erythrocytes, spherocytes,	anisopoikilocytosis, abundant spherocytes

Table 1.

Conclusion:

Despite EMA binding test has a sensitivity of 92.7% and a specificity of 99.1% for HS, other membranopathies can also display EMA detriment as we observed in our study. If this test is considered isolated it could lead to a misdiagnosis with clinical consequences, thus, other aspect such as clinical manifestations, RBC morphology hematimetric parameters and molecular studies (only necessary in selected cases) must be considered to reach a correct diagnosis.

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Optimization and standardization of nuclear Ki67 staining by flow cytometry and its utility in plasma cells neoplasms

Luzalba Sanoja-Flores[1], Juan Flores-Montero[1], Vítor Botafogo[1], Noemí Puig[2], JJM van Dongen[3], Brian Durie[4], Julia Almeida[1], Alberto Orfao[1]

[1]Universidad De Salamanca, Salamanca, España, [2]Department of Hematology, University Hospital of Salamanca, IBSAL; IBMCC (USAL-CSIC), Salamanca, España, [3]Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, The Netherlands, [4]Cedars-Sinai Samuel Oschin Cancer Center, Los Angeles, United Stated of America

Introduction:

Ki67 is a nuclear (n) protein expressed throughout the cell-cycle once G_1 starts, reflecting the actively proliferating cell compartment. Because of its localization, aggressive permeabilization protocols are frequently used and their performance is often suboptimal. A high plasma cell (PC) proliferation is a well-known adverse prognostic factor in plasma cell neoplasm (PCN) but assessment of nKi67 by flow cytometry is limited. Overall, we aim at optimizing and standardizing the evaluation of Ki67 expression to identify proliferative bone marrow (BM) PCs from healthy donors (HD) and PCN patients at diagnosis.

Methods:

The manufacturer recommended protocol (based on 70% ethanol and freezing) was evaluated in 1 BM sample while i) *Fix&Perm (Nordic-MUBio BV)-based permeabilization and ii)* an adapted method to assess *transcription factors* (TF; eBioscience) were compared in 5 BM samples. Thereafter, a total of 5 HD, 8 gammopathy of undetermined significance (MGUS) and 6 multiple myeloma (MM) patients (5 smolderingMM and 1 symptomaticMM) were processed using the optimized protocol to identify proliferative BM-normal (NPC) and tumor PC (TPC) and other (residual) BM cell populations using a FACSCantoII cytometer (BD Biosciences) and the *Infinicyt* software (version 2.0; Cytognos) for data analysis.

Results:

Manufacturer recommended protocol resulted in nKi67+ staining in 27% of cells in the sample, but cells were severely affected by the protocol (≥99% dead cells) hampering the analysis. In turn, *Fix&Perm*-based protocol kept normal BM cell characteristics but failed to detect any nKi67+ cells. In contrast, the adapted TF method, allowed detection of nKi67+ cell compartment without compromising counterstaining and/or light scatter properties, allowing for an accurate discrimination of BM-NPC and -TPC and other residual cells present

in the sample in all cases (13/13, 100%). Thus, the percentage of nKi67+ NPC was progressively increased from HD (median 3.7%, range: 2.2%-4.6%) to MGUS (median 8.7%, range: 2.1%-64.9%) and MM patients (median 11.3%, range: 3.5%-80.8%) (p=0.03 HD vs MM). Among TPC, we observed no significant differences in the percentage of TPC nKi67+ from MGUS (median 2.1%, range: 0.6%-11.3%) to MM patients (median 3.5%, range: 1.5%-18.9%).

Conclusions:

The optimized TF method is a useful approach to identify BM nKi67+ cells while preserves the normal BM cell characteristics and distribution by flow cytometry. Moreover, the use of this technique in PCN patient samples detect increased proliferation rates in NPC associated with more advanced disease categories. Clinical utility of this method to identify nKi67+ populations in other diseases and sample types deserve further investigation.

14

Comparison between flow cytometry and pathological anatomy as diagnostic techniques in FNA guided by EBUS

Nahir Daniela Moreno Paredes[1], Ana María Gonzalez Salinas[2], Alberto López García[3], Melisa Almendra Daorta[4], Roberto Oswaldo Trelles Martínez[5], Estefanía Bolaños Calderón[6], Fernando Ataulfo González Fernandez[7], Sissy Fiorella Medina Salazar[8], Elena Forcen[9], María José Bernabé[10], Melchor Saiz-Pardo Sanz[11], María José Fernández-Aceñero[12], Rafael Benigno Martínez Martínez[13], Celina María Benavente Cuesta[14]

[1]Hospital Clínico San Carlos, Madrid, España, [2]Hospital Clínico San Carlos, Madrid, España, [3]Hospital Clínico San Carlos, Madrid, España, [4]Hospital Clínico San Carlos, Madrid, España, [5]Hospital Clínico San Carlos, Madrid, España, [6]Hospital Clínico San Carlos, Madrid, España, [7]Hospital Clínico San Carlos, Madrid, España, [8]Hospital Clínico San Carlos, Madrid, España, [9]Hospital Clínico San Carlos, Madrid, España, [10]Hospital Clínico San Carlos, Madrid, España, [11]Hospital Clínico San Carlos, Madrid, España, [12]Hospital Clínico San Carlos, Madrid, España, [13]Hospital Clínico San Carlos, Madrid, España, [14]Hospital Clínico San Carlos, Madrid, España

Introduction:

Flow Cytometry (FCM) is a useful diagnostic tool in the study of hematological pathologies. Although its isolated application does not allow to establish a diagnosis certainty, the rapidity to obtain results makes this technique more and more used, even in non-hematological pathology. In order to evaluate the diagnostic value of the FCM in FNA (fine needle aspiration) samples guided by EBUS (endobronchial ultrasound) we considered the need to compare FCM with the Pathological Anatomy (PA) to determine if there is a correlation between both techniques and in this way establish the utility of the FCM as a diagnostic tool.

Methods:

A retrospective observational study was developed in our center, in which 30 FNA samples guided by EBUS of mediastinal adenopathies or lung masses obtained during the period from January 2016 to January 2019 were compared. The samples were processed by FCM and PA. The statistical analysis was carried out using Cohen's kappa coefficient. Three variables were analyzed: normal / reactive result, lymphoproliferative syndrome (LPS) and non-hematological neoplasia. A $p < 0.05$ was considered as a statistically significant result.

Results:

In the normal / reactive variable (20 cases) there was an agreement between both techniques of 90%, FCM 20/20 versus PA 18/20. In PA the remaining 2 cases were identified as suggestive of LPS requiring a third diagnostic technique, in which the diagnostic

probability was not confirmed. It must be noted that 1 of the cases, in which the result was inconclusive for both techniques, later a third analysis was a diagnosed as Hodgkin lymphoma (HL). In the SLP variable (3 cases) there was an agreement of 100%. In the case of non-haematological neoplasias (7 cases) there was an agreement of 85.7%, FCM 7/7 versus PA 6/7. For PA, the remaining case was a negative false of adenocarcinoma identified by a third diagnostic technique. By Kappa de Cohen a degree of agreement of 0.806 was obtained with a $p < 0.01$.

Conclusion:

The agreement degree between these two techniques suggests that the FCM has a diagnostic profitability similar to or superior to PA in FNA samples guided by EBUS, being complementary techniques that should be performed simultaneously, which facilitates a quick diagnostic orientation, directing the decision about patient management.

15

Comparative analysis of different photo illumination systems for extracorporeal photopheresis

Lourdes Cordón[1,3], Carlos Carretero[1,2], Marta Santiago[3], Pilar Solves[2,3], Amparo Sempere[2,3], Lidia Navarro[2], Dolores Moreno[2], Maribel Bartual[2], Alejandra Sanjuan[1], Esperanza Such[1,2,3], Guillermo Sanz[2,3], Inés Gómez-Seguí[2,3]

[1]Grupo de Investigación en Hematología - Instituto De Investigación Sanitaria La Fe, Valencia, Spain, [2]Servicio de Hematología y Hemoterapia - Hospital Universitario y Politécnico La Fe, Valencia, Spain, [3]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC) - Instituto Carlos III, Madrid, Spain

Introduction:

Extracorporeal photopheresis (ECP) is a treatment consisting in the collection of patient's lymphocytes, its photo inactivation (PIN) using psoralen (8-MOP), exposure to UV-light, and reinfusion of the treated cells to the patient. This process induces the lymphoid apoptosis and modulates the altered immune response in graft versus host disease (GvHD) or cutaneous T-cell lymphoma. Among different photo illumination systems, it is not well known which the most efficient inducing apoptosis is. This study aimed to validate the photo-illumination process in UVA-PIT (Med Tech solutions) and Macogenic G2 (Macopharma) systems, comparing the viability and the apoptosis in photo inactivated apheresis products.

Methods:

A total of 10 ECP treatments from patients diagnosed of chronic GvHD (n=8), acute GvHD (n=1), and necrobiotic xanthogranuloma (n=1) were included. The mononuclear cells were collected in a Spectra Optia. The product was photo-illuminated after addition of 8-MOP, 4 in the UVA-PIT and 6 in the Macogenic G2 system, either at standard (300mL; n=3) or at reduced volume (200mL; n=3) for paediatric patients. A pre- (before 8-MOP addition) and a post-illumination sample from product was obtained and studied by flow cytometry (FC) after 4 h. A total of $5x10^6$ cells from both samples were cultured (supplemented RPMI, 37ºC, 5% CO_2) and then analysed after 48 h. Viability (propidium iodide, PI) and apoptosis (annexin-V-FITC) were measured by FC at 4 time points (pre- and post-PIN, and pre-PIN and post-PIN after 48 h cultured). Kruskal-Wallis test was used for the statistical analysis.

Results:

Median cellularity of products was $85x10^6$ leukocytes/Kg (range 17-$143x10^6$), being 61% lymphocytes (range 33-83). The median haematocrit in the product was 1,5% (range 0,6–

2,2). Apoptosis was classified as early, late or necrosis. The cell viability diminished in both photo illumination systems compared with the pre-inactivated sample (median of 2,4% at 4 h and 31% at 48 h). The Macogenic system at standard volume provided higher decrease in viability and greater necrosis, although differences were not statistically significant.

Conclusion:

The three PIN processes in both systems were valid for ECP in terms of cell death induction and reduction of viability. The low apoptosis rate observed could be justified by the time lapsed (48 h), with a high percentage of established necrosis in the samples. Although no statistically significant differences were observed between PIN systems, it might be due to the low number of cases.

Acknowledgements:

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Progression of a T-lymphoblastic lymphoma to an acute T-cell leukemia with aberrant expression of the B-cell antigen CD19

Tamara Mateu-Albero[1], Ligia Gabriela Gabrie Rodríguez[1], Laura Esparcia-Pinedo[1], Ildefonso Sánchez-Cerrillo[1], Ana García-Noblejas[2], Victor López-Huete[1], Cecilia Muñoz-Calleja[1]
[1]Immunology Department - La Princesa University Hospital, Madrid, Spain, [2]Hematology Department - La Princesa University Hospital, Madrid, Spain

Introduction:
T-Lymphoblastic Lymphoma (T-LBL) is a rare form of aggressive non-Hodgkin's lymphoma which represents 85-90% of all LBLs. The most common form of presentation is a mediastinal mass, and usually with <25% of bone marrow (BM) involvement. T-LBL is composed of immature lymphoblasts committed to T lineage that express cytoplasmatic CD3 (cytCD3), CD7 and TdT, with variable expression of others T-associated markers.

Clinical case presentation: We present a case report of 50-year-old woman with night sweats and nonpruritic erythematous and violaceous rash on the mammarian region during the last 3-months. A chest abdomen pelvis computed tomography revealed small paratracheal adenopathies and an anterior mediastinal mass. No other signs or symptoms were found.

Definitive diagnosis: Morphological and histological analysis of both the skin and the mediastinal mass confirmed the suspicion of T-LBL with cutaneous involvement. Immunohistochemistry the blast cells were CD3+, TdT+, CD7+, CD99+, CD79a- and Ki67+ (>90%). Neither the expression of CD19 nor Pax-5 were investigated. The flow cytometry (FC) analysis of both peripheral blood (PB) and BM revealed no infiltration.

Treatment and Evolution: She was treated first with the PETHEMA ALL-AR-2011 and FLAG-Ida regimen, but both treatments failed. An allogeneic HLA-identical sibling-donor BM transplant and a mastectomy were performed followed by nelarabine maintenance treatment and a sibling-donor lymphocyte infusion, resulting in a complete remission (CR). Two years later, after multiple relapses and different failed treatments, the patient rapidly progressed to a frank acute leukemia and, this time, tumoral T lymphoblasts could be detected by flow cytometry in PB. The proportion of these tumoral cells increased from 4% of the leukocytes to >40% in 9 days. Molecular biology analysis confirmed the clonal rearrangement of the T-cell receptor gamma chain gene. BM was not made.

Immunophenotypic strategy and results: An 8-color FC immunophenotyping panel was performed. The tumoral T-cells were CD45dim, TdT+, CD3+, cytCD3+, CD7+, CD5dim, CD2-, CD4-, CD8-, CD99-, CD33+, CD38+, HLA-DR- and Ki67+ (25%). The FC study also confirmed the expression of CD79a and interestingly, the B-linage specific marker CD19 was strongly expressed although no other B-associated markers were positive.

Discussion: This is an extraordinarily exceptional case of a T-LBL with aberrant expression of CD19. It is likely that the different treatment regimens may have caused cytogenetic alterations that promoted the expression of CD19. Other reasons may include a Pax-5 deficiency which has been demonstrated to cause B-cell-derived T cells.
Taking into account the fateful prognosis of T-LBL and the promising therapies against CD19-expressing neoplasms, including bispecific antibodies and CAR T cells, we therefore encourage the analysis of the expression of CD19 both at diagnosis and in every relapse.

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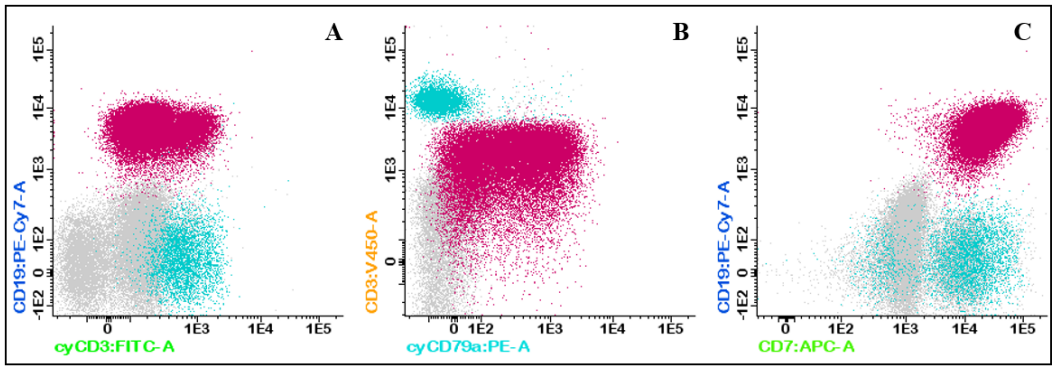

Figure 1. Immunophenotypic analysis of T-LBL (pink) showing the expression of CD19-cytCD3 (A), CD3-cyCD79a (B) and CD7-CD19 (C). Normal T cells are coloured in blue.

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Biclonal lymphoproliferative disease versus transformation: report of a clinical case

Patricia Amantegui[1], Mafalda Felgueiras[1], Andreia Monteiro[1], Paula Gameiro[2], Patricia Sousa[1]
[1]Centro Hospitalar Universitário Cova Da Beira, Covilhã, Portugal, [2]Instituto Português de Oncologia Lisboa, Lisboa, Portugal

Waldenström macroglobulinemia (WM) is a B cell lymphoproliferative disease characterized by the accumulation of monoclonal cells in the bone marrow and peripheral lymphatic tissues associated with the monoclonal production of immunoglobulin M (IgM) in the serum, representing 2% of all hematological malignancies. In 5% to 10% of the cases, patients with WM develop B-cell Diffuse Large Hodgkin's Lymphoma (DLBCL) as histological transformation.

This report describes the case of a patient with atypical clinical presentation with simultaneous diagnosis of WM and DLBCL.

A 54-year-old male with a history of smoking, chronic obstructive pulmonary disease and depressive syndrome, referred to a clinical presentation, with two months of evolution characterized by asthenia and exertional dyspnea, associated with evening fever and weight loss of 5kg in 2 months. Upon admission, a massive right pleural effusion is associated with mild microcytic anemia and elevated serum ADA, LDH and CRP levels, the diagnostic hypothesis of pleural tuberculosis has been established. The immunophenotypic study (IF) of pleural fluid documents an infiltration into 51% kappa monoclonal plasmocytes (CD38, CD138 positive). The biochemical study confirms the presence of paraprotein, IgM kappa of> 50 g / L. The peripheral blood smear (PBS) identifies erythrocyte rouleaux, 4% plasma cells, and polymorphous lymphocytes. The venous blood IF study confirms 4% of kappa monoclonal plasmocytes (identical to that identified in pleural fluid) and 12% of kappa monoclonal B cells, with the hypothesis of one WM. A bone marrow biopsy is performed to confirm the diagnosis, and the MYD88 L265P mutation is negative. For refractory pleural effusion, the patient performs a right thoracoscopy, with visualization of parietal and visceral pleural multifocal nodular lesions, followed by right pleurodesis. The pleural fluid cytology is diagnostic of small cell LNH B and the biopsy of the parietal pleura of a DLBCL. The patient is referred to the Hematology Service, initiating curative chemotherapy, directed to aggressive lymphoma, with RCHOP protocol.

The clinical evolution of WM patients is indolent (median survival 60 months). However, there is a risk of histological transformation to more aggressive lymphoma subtypes, which may occur at any time during the course of the disease, with the rate of transformation of WM to DLBCL being about 2.4% at 10 years. In these situations, the course of the disease becomes more aggressive and with a worse prognosis than in a *de novo* DLBCL (median survival of 2 to 5 months).

Given the simultaneity of the two diagnoses, and after the study of the rearrangement of the immunoglobulins by the PCR amplification technique and Genescanning analysis, a clonal rearrangement was detected for the heavy chain genes (complete rearrangement, type VH-JH) and a rearrangement clonal expression for the kappa light chain genes (VK-JK type rearrangement) of immunoglobulin, identical in both samples, bone marrow and pleural biopsy.

After 3 cycles of RCHOP the patient is asymptomatic, ECOG 0 with 80% reduction of the monoclonal protein.

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Reconstitution of antigen presenting cells (B lumphocytes, neutrophils, monocytes and dendritic cells) after allogeneic hematopoietic transplantation in 15 adults

Agustín Penedo Coello[1], María Sánchez[1], Y. Jiménez[1], R. Gonzalo[1], S. Castañón[1], S. Ledesma[1], Juan Manuel Alonso[1], José Luis López Lorenzo[1], Cristina Serrano[1]
[1]*Fundación Jiménez Díaz Hospital, Madrid, Spain*

Introduction:

B cell and other antigen-presenting cells (APCs) reconstitution is important for the hematopoietic postransplant recovery of immune response capacity. We describe the evolution of APCs reconstitution (lymphocyte B subpopulations, plasmacytoid dendritic cells (PDC), neutrophils and monocytes) as well as evolution of immunoglobulins (Igs) levels in a group of patients treated with allogeneic hematopoietic cell transplant (allo-HCT).

Methods:

Fifteen adult (>18 years) patients with hematological malignancies treated with allo-HCT from May 1st to December 31, 2018 in a terciary level university hospital.

Flow cytometry was performed in peripheral blood at days 0, +15, +30, +100, +180 after transplantation. Specific monoclonal antibodies were used for the surface membrane immunoglobulin D (IgD), CD27, CD19, CD45Ra, CD38, CD4, CD3. The immunophenotype data were analized using Infinicity^{TM} software (Cytognos). The data was resumed in tables.

Results:

- PDC were detected from day + 30 (median 3.5 cel/mcL, range 0-27), with stable levels since then.

- Neutrophils progresively diminished from day 0 with lower level at day +15 (median 200 cel/mcL, range 0-3900). They were raising progresively until normal level at day +30 (median 1600 cel/mcL, range 600-6400).

- Monocytes behaivor was similar to neutrophils.

- B lymphocytes population was scarce at day 0 (median 2 cel/mcL, range 0-234) with 68.92% CD19+IgD+CD27- (naïve) immunophenotype. From day +100 B lymphocyte populations were identified as CD19+IgD+CD27- (96.6%, median 54 cel/mcL, range 0-232) and CD19+IgD-CD27+ (switched-memory-B-cells) (0.6%, median 0.5 cel/mcL, range 0-1).

They raised progresively until day +180 with a median total B lymphocyte population of 142 cel/mcL (range 32-143). At day +180 naïve B lymphocytes represents 88% of total (median 124, range 31-124) and aditional B lymphocyte populations were identified with phenotype CD19+IgD+CD27+ (non-switched-memory-B-cells)(0.63%, median 1, range 0-1) and CD19+CD27+CD38+ (pre-plasmatic-cells)(2.52%, median 4, range 0-4).

IgG and IgM were under inferior normal limit at day 30 but rised to normal at day 100. IgA were under inferior normal limit at day 100 but rised to normal at day 180.

Conclusion:

PDC, neutrophils and monocyte population recovered early in the postransplant period. There are few B cells before alo-HCT, but they reach normal counts after day +100 with presence of memory cells and pre-plasmatic-cells. Individualized study of APC reconstitution could be used as an indicator of vaccination program after allo-HCT.

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Altered immunophenotypes on leukemic or monocytic cells from acute myeloid leukemia highly predict for nucleophosmin gene mutation

Sergio Matarraz[1,8], Rosa Ayala Bueno[1,8], Luis García Alonso[2], Xavier Calvo[3], Enrique Colado[4], Jose Ignacio Sánchez-Gallego[1,8], Vincent van der Velden[5], Pilar Leoz[6,8], María Belén Vidriales[6,8], Ramón García Sanz[6,8], Maria Carmen Chillón[6,8], Neus Villamor[7], Sara Alonso[4], Ariana Fonseca[4], Covadonga Quirós Caso[4], Félix López Cadenas[6,8], María Díez-Campelo[6,8], Leonor Arenillas[3], María Isabel Prieto Conde[6,8], Laura Magnano[7], Jose Antonio García Vela[2], Carlos Fernandez[1,8], Andrea Mayado[1,8], Daniela Damasceno[1,8], Susana Barrena[1,8], Antonio López[1,8], Alberto Orfao[1,8]

[1]Cytometry Service. Centro De Investigación Del Cáncer (ibmcc, Csic-university Of Salamanca), Salamanca, Spain, [2]Hematology Service, University Hospital of Getafe, Getafe, Spain, [3]Pathology Service, Hospital del Mar, Barcelona, Spain, [4]Central University Hospital of Asturias, A.G.C. Hematology Laboratory, Oviedo, Spain, [5]Department of Immunology, Erasmus MC, University Medical Center Rotterdam, Rotterdam, The Netherlands, [6]Hematology Service, University Hospital of Salamanca and IBSAL, Salamanca, Spain, [7]Hematology Service, Hospital Clinic, Barcelona, Spain, [8]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain

Introduction:

Nucleophosmin gene mutation (*NPM1^{mut}*) occurs in around 30% of acute myeloid leukemia (AML) patients, frequently with favourable prognosis. Despite the expanding knowledge on the clinical impact of *NPM1^{mut}* and its associated molecular background (e.g. *FLT3-ITD^{mut}*), more expeditious diagnostic approaches are needed for early therapy intervention. Herein, we investigated the association of immunophenotypic features of leukemic and monocytic cells with the presence of *NPM1^{mut}* in AML.

Methods:

Bone marrow (BM) leukemic and monocytic cells from 292 AML patients were studied by 8-color flow cytometry using the EuroFlow AML panel. *NPM1^{mut}* was present in 223 patients, whereas remaining 69 cases showed wild type gene (*NPM1^{wt}*). Presence of *FLT3-ITD^{mut}* was found in 42 of 212 cases studied (20%), while concomitant *NPM1/FLT3^{mut}* was observed in 29/42 cases (69%).

Results:

Leukemic cells with monocytic differentiation were observed in 58% vs. 42% of AML with *NPM1^{mut}* vs. *NPM1^{wt}* cases. In those cases, lacking monocytic differentiation traits on leukemic cells, remaining monocytic cells were similarly studied. Overall, abnormal monocytic patterns were more recurrent among *NPM1^{mut}* vs. *NPM1^{wt}* cases, both on leukemic cells (98% vs. 38%) and monocytic cells (76% vs. 25% of cases; p<0.001). Most frequent abnormal phenotypic patterns among *NPM1^{mut}* leukemic cells consisted of asynchronous CD35 vs. CD14 (86% vs. 27%) and/or CD300e vs. CD14 expression (78% vs. 15% *NPM1^{wt}* cases, respectively; p<0.001), independently of *FLT3-ITD* comutation. Noteworthy, coexistence of both asynchronous monocytic patterns was more frequent

on *NPM1*^{mut} vs. *NPM1*^{wt} patients (67% vs. 6%; p<0.001). Interestingly, however, two opposite CD35/CD14 asynchronous monocytic patterns were observed. Thus, CD35 was acquired before CD14 in 96% vs. 25% of *NPM1*^{mut} vs. *NPM1*^{wt} cases, while CD14 was more often acquired before CD35 in *NPM1*^{wt} cases (4% vs. 75%, respectively; p<0.001). Of note, monocytic cells from *NPM1*^{mut} AML without monocytic differentiation showed both asynchronous patterns in 45% vs. 7% of *NPM1*^{wt} cases (p<0.001).

Finally, CD9 aberrant leukemic cell expression was more frequent in AML with either isolated *NPM1*^{mut} or *FLT3-ITD*^{mut}, and also among *NPM1/FLT3-ITD*^{mut} cases (68%, 75% and 92% vs. 45% of *NPM1/FLT3-ITD*^{wt} cases, respectively; p≤0.03). Noteworthy, simultaneous expression of CD9 together with both asynchronous (CD35/CD14 and CD300e/CD14) leukemic cell patterns was restricted to *NPM1*^{mut} AML (60% vs. 0% *NPM1*^{wt} cases; p<0.001), while aberrant CD25 was otherwise linked to *FLT3-ITD*^{mut} (73% vs. 18% of *FLT3-ITD*^{wt} cases; p<0.001) and not *NPM1*^{mut} AML.

Conclusions:

The presence of asynchronous monocytic patterns among leukemic or monocytic cells from AML patients, together with aberrant CD9 leukemic cell expression is highly predictive for *NPM1*^{mut} and may contribute to upfront therapy selection in AML.

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Dissection of the normal blood B-cell compartment in CLL and MBL: evidence of alterations at early stages of pre-leukemic conditions

Ignacio Criado[1], Elena Blanco[1], Arancha Rodríguez-Caballero[1], Miguel Alcoceba[2], Teresa Contreras[3], María Laura Gutiérrez[1], Alfonso Romero[4], Paulino Fernández-Navarro[5], Marcos González[2], Fernando Solano[6], Carlos Gómez[6], Martín Pérez-Andrés[1], Jacques J.M. van Dongen[7], Julia Almeida[1], Alberto Orfao[1]

[1]Cancer Research Center, University of Salamanca, CIBERONC and IBSAL, Salamanca, Spain, [2]Hematology Service, University Hospital of Salamanca, IBMCC, CIBERONC, IBSAL, Salamanca, Spain, [3]Biochemistry Service, University Hospital of Salamanca, Salamanca, Spain, [4]Centro de Atención Primaria Miguel Armijo, Sanidad de Castilla y León (SACYL), Salamanca, Spain, [5]Centro de Atención Primaria de Ledesma, Sanidad de Castilla y León (SACYL), Salamanca, Spain, [6]Hematology Service, Hospital Nuestra Señora del Prado, Talavera de la Reina, Spain, [7]Department of Immunohematology and Blood transfusion, Leiden University Medical Center, Leiden, Netherlands

Introduction:

Monoclonal B-cell lymphocytosis (MBL) and chronic lymphocytic leukemia (CLL) are both associated with higher risk of infections compared to the general population, pointing out an underlying altered immune response already present at the very early disease stages. However, little is known about the mechanisms and cell populations involved. Here we aimed to analyze in detail the normal peripheral blood (PB) residual B-cell compartments and antibody plasma levels of both low- and high-count MBL (MBL^{lo} and MBL^{hi}) and Binet stage A/Rai 0 CLL vs. non-MBL controls.

Methods:

PB B-cell subpopulations and antibody plasma levels of all the different immunoglobulin (Ig) subclasses were measured in a total of 110 cases, distributed into four study groups: MBL^{lo} (n=27), MBL^{hi} (n=21), early stage CLL patients (n=22) and non-MBL sex- and aged-matched healthy donors (controls, n=40), using high-sensitivity 12-color flowcytometry and nephelometry, respectively.

Results:

Residual normal PB B-cell counts were reduced in both MBL^{hi} and CLL (vs controls), mostly due to a decreased production of immature and naïve B cells; conversely, normal numbers of total memory B cells (MBC) and plasma cells (PC) were observed in CLL, together with lower PC numbers in MBL^{hi}. Despite this, a progressively altered distribution of MBC and PC expressing distinct Ig-subclasses was found, consisting of: decreased IgM^+ PC in MBL^{lo}, lower

PC counts of all Ig-subclasses in MBL^{hi}, and decreased numbers of MBC and PC expressing Ig-subclasses and antibody plasma levels encoded downstream in the IGHC gene (i.e. IgG2, IgG4, IgA2) in CLL.

Conclusions:

A reduced release of pre-germinal center B-cells into PB already occurs in an early stage (i.e. MBL), which might narrow the B-cell repertoire and progressively would hamper the production of antigen-experienced B-cells at CLL stage A, with a progressively more marked secondary immunodeficiency and greater risk of infection. Further studies are required to confirm this hypothesis, and to better understand the role of an impaired immune system at the very early stages of pre-leukemic conditions in the progression to an overt CLL.

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Bone Marrow Haemodilution Assessment in Flow Cytometry

Sara Braga[1], Ana Torgal[1], João Pedro Barreto[1], Cátia Mendes[1], Cátia Rodrigues[1], Marta Pires[1], Carla Azevedo[1], Inês Godinho[1], Emília de Sousa[1], Carlos Palmeira[1], Gabriela Martins[1]
[1]*Instituto Português de Oncologia do Porto, Francisco Gentil, E.P.E., Porto, Portugal*

Introduction:

Bone marrow aspirates for Flow Cytometry (FC) analysis are usually obtained from a second aspiration, the first being used for morphological assessment. That's why they almost unavoidably contain some peripheral blood (PB) contamination.

The quality evaluation of samples for FC is of greatest importance, to assure they are representative, allowing a more accurate interpretation of results, especially for the study of Minimal Residual Disease (MRD).

However, PB contamination is not evaluated in most laboratories, because methods available are either qualitative or too complex and time consuming.

The present work applied a quantitative method to evaluate haemodilution in two Multiple Myeloma (MM) cases where MRD evaluation by FC was negative despite the disease being detected by another method, raising doubts, since FC is supposed to be more sensitive than Immunofixation (IF) or Electrophoresis (EF) and neither of the patients had a detectable secreting plasmacytoma.

Methods:

Case 1:

MM IgA kappa, in complete remission where IF revealed an IgA kappa monoclonal band.

Case 2:

MM IgG kappa, in partial remission where EF revealed a quantifiable IgG kappa monoclonal peak of 7.37g/L.

On both cases the second portion of a BM aspirate was received on the 60th day post-autotransplant for MRD detection by FC. No pathological plasma cells were detected in either sample (> $5x10^6$ cells were acquired).

A quantitative method was used for haemodilution assessment, based on the percentage of plasma cells (PC), CD34+ cells and CD10+ granulocytes: Peripheral Blood Contamination Index (PBCI)= $-3.052+0.065\times(\%CD10^{+}G) -0.609\times(\%CD34^{+}) -2.008\times(\%PC)$.

PBCI >1.2 discriminates low quality samples.

Results:

According to the authors, if the disease physiopathology induces abnormal values for one parameter, the PBCI is calculated with the remaining two and the validity of the threshold is maintained. Here the plasma cell percentage was, therefore, excluded.

Case 1: PBCI=1.74

Case 2: PBCI=3.11

Both samples were henceforth considered of low-quality and inadequate for MRD evaluation.

Conclusions:

These cases highlight the need for a measurable criterion of PB sample contamination assessment and for good quality sample collection for FC analysis, namely, the 1st tap of the marrow aspirate, as recommended by Euroflow Guidelines.

The PBCI method was chosen over a few others described in literature since it was found to be the most objective, reproducible and of easiest application (only CD10 and CD34 markers must be added to the Euroflow MM-MRD antibodies panel), providing a quantitative assessment of PBCI and a specific cut-off to guide decision-making.

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Comprehensive study of mature proliferating B-cells in clinical samples with suspected lymphoma

Dolores Subirá[1], Fabiola Barriopedro[1], Ruth Martínez[1], Nuria Golbano[1], Alejandro Vázquez[1], Miguel Díaz-Morfa[1], Dunia de Miguel[1]
[1]*Hospital Universitario De Guadalajara, Guadalajara, Spain*

Introduction:

Measurement of the S and G2M-phases of cell cycle reflects cell growth speed and helps to discriminate between aggressive and indolent B-cell lymphoma. B-lymphocytes complete their maturation in lymph nodes, so some reactive conditions may also exhibit a high proliferative activity. We have explored the significance of the B-cell cycle in samples sent to rule out lymphoma.

Methods:

From 2016 to 2018, 112 samples were sent for standard flow cytometry (FC) analyses to discard lymphoma. A cell cycle was performed in 59 samples (58 patients) which had ≥5 x10^6 viable cells necessary for this assay. Most samples (n=49) were biopsies from lymph nodes, followed by 7 extranodal sites, 2 pleural effusions and 1 ascitic fluid. Histopathology established the definitive diagnosis. The FC cell cycle protocols included staining of surface antigens and measurement of nuclear DNA content using either propidium iodide (n=2), DAPI (n=50) or DRAQ5 (n=7). At least, 2 x 10^6 events were acquired on a FACSCanto II flow cytometer. The Infinicyt software program was used for analysis and quantification of cells in the G0/G1, S and G2M-phases.

Results:

Samples were diagnosed with 16 low-grade B-NHL (50% follicular lymphoma (FL) grade 1-2), 12 FL grade 3a (FL3a), 23 aggressive B-NHL (78.3% diffuse large B-cell lymphoma), 4 T-NHL, 3 reactive lymphadenitis and 1 Epstein-Barr virus explosive follicular hyperplasia (EBV-EFH). The mean percentage of neoplastic B-cells in the S/G2M-phases was 1.41%±1.66 in low-grade B-NHL, as compared to 2.23%±2.4 in FL3a (p>0.05) and 22.8%±20.36 in aggressive B-NHL (p<0.001). No statistically significant difference was observed between FL grade 1-2 and FL3a. The range of cells in the S/G2M-phases in patients diagnosed with aggressive B-NHL was very wide (0.9-82.3%). Interestingly, 2 additional patients (EBV-EFH and angioimmunoblastic T-NHL) had a clonal B-cell population (14% of cells) and a high

percentage of them were in the S/G2M-phase (23.4% and 28% respectively). The mean percentage of polyclonal B-cells in S/G2M-phases was 0.83%±0.43 in reactive lymphadenitis and 1.62%±1.96 in the 3 remaining T-NHL. A cut-off value of >3% S/G2M cells identified aggressive B-NHL with a sensitivity, specificity, positive and negative predictive value of 86.95%, 83.3%, 76.92% and 90.9%, respectively.

Conclusions:

A high fraction of monoclonal B-cells in the S/G2M-phase predicts aggressive B-NHL. However, EBV-positive B-cell proliferations should also be considered in the differential diagnosis. On the other hand, an inadequate representative sample for FC evaluation might explain the low proliferative index seen in some aggressive lymphoma.

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Evolution of essential thrombocythemia into acute leukaemia with concurrent erythroid and megakaryocytic differentiation

Teresa Caballero-velázquez[1], Rosario Morales-Franco[1], Olga Pérez-López[1], Concepción Prats[1], María Teresa Vargas[1], Ricardo Bernal[1]

[1]nstituto de Biomedicina de Sevilla, UGC de Hematología, Hospital Universitario Virgen del Rocío and Consejo Superior de Investigaciones Científicas (CSIC) and Centro de Investigación Biomédica en Red Cáncer (CIBERONC), Universidad de Sevilla, Seville, Spain., Sevilla, Spain

Introduction:

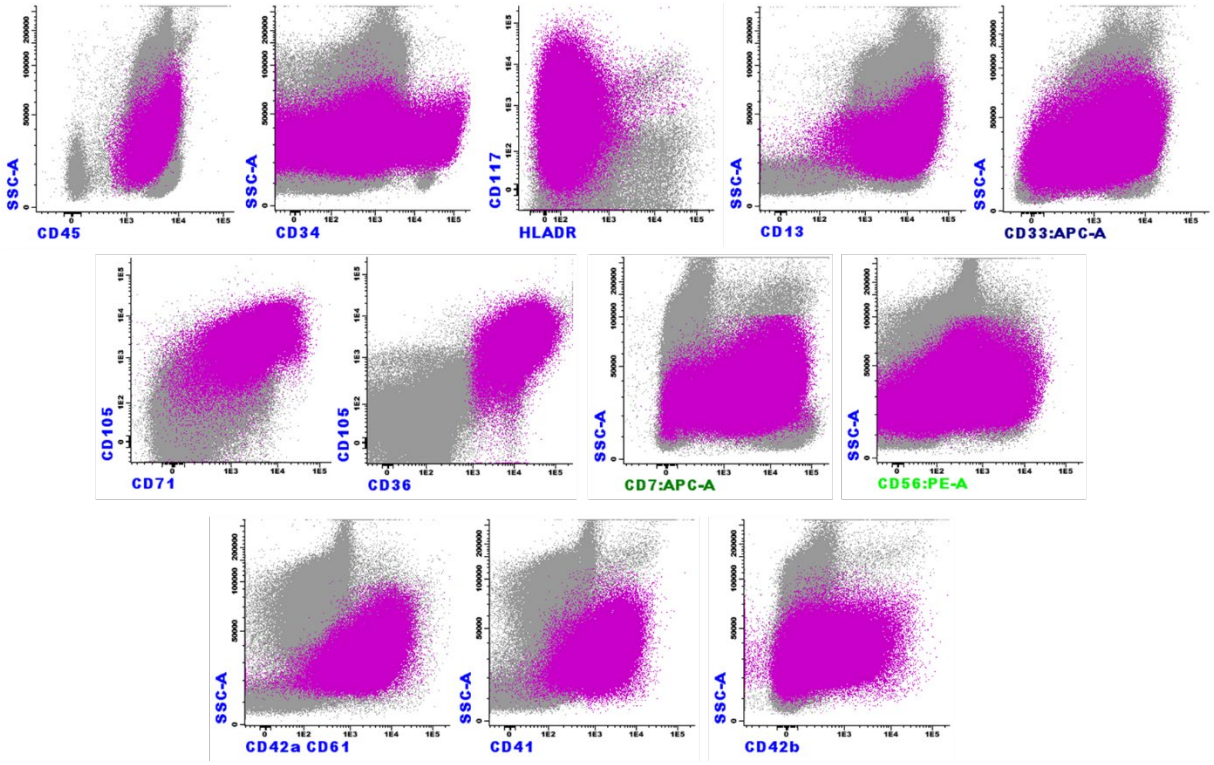
Essential Thrombocythemia (ET) is a *BCR-ABL1*-negative myeloproliferative neoplasm characterized by thrombocytosis with a tendency towards thrombosis and haemorrhage. Lesser patients transform to myelofibrosis or acute myeloid leukaemia (AML) as shown in the IPSET study where 891 patients with ET with 15-year cumulative risk of AML was 2 percent. Progression time to acute leukaemia from diagnosis is usually more than 10 years. We report the case of 71-year-old man who developed an acute leukaemia after 20 months of treatment of hydroxyurea treatment for his ET.

Case:

A patient with a history of diabetes and dyslipemia was referred to the department of haematology. Blood count revealed thrombocytosis (869 x10e9/L), Hb of 139 g/L and leukocytes of 11,26 x10e9/L. JAK2 in peripheral blood was positive in 50% of cells. The result of bone marrow aspirate and biopsy was ET. No fibrosis or iron deposits were seen in the biopsy. Acetyl salicylic 100 mg per day and hydroxyurea 1 gr per day were started. After 20 months of follow-up, the patient with a one-week fever came to the emergency department, did not respond to antibiotic treatment. Blood count showed anaemia (97 g/L), thrombocytopenia (47 x 10e9/l) and leukocytes of 10,68 x 10e9/l (neutrophils 6.6 x10e9/l and monocytes 1.8 x10e9/l). Twenty-nine percent of medium and large-sized blast cells were observed in the peripheral blood smear. Progression to AML was suspected. Bone marrow aspirate was hypercellular with 76,8% blast cells. Blast cells were positive to PAS and acid phosphatase 30% and 100%, respectively. At the same time, flow cytometry analysis was performed. Firstly, Acute Leukaemia Orientation Tube (ALOT) according to Euroflow panels was evaluated. Sixty five percent of blast cells was identified with the following immunophenotype: CD34 -/+(7%) CD45+ CyMPO- CD19- CyCD79a-, CyCD3-SmCD3- and CD7+. According to this result, tubes 1-6 Euroflow AML panel were processed. Blast population was CD33-/+het CD13+het HLADR- CD4+/- CD9- CD38+ CD35+ CD36+ CD71+ CD105+ CD123-/+ CD7+het CD56-/+ CD61/CD42a+ and negative for the rest of markers. Seventh tube of Euroflow AML and another tube with CyCD61 and CD45 were acquired. Only one population expressed erythroid (CD36+ CD71+ and CD105+) and megakaryocytic markers (CyCD61+ SmCD61/CD42a+ CD41+ and CD42b-/+(36%) CD36+). Cytogenetic study showed a complex karyotype with a hypertriploid (70-80 chromosomes). The patient passed away three days after diagnosis due to pulmonary infection. Discussion: ET can evolve to secondary acute myeloid leukaemia (<5%) (sAML), which has a very poor

prognosis. Acute erythroid leukaemia and acute megakaryoblast leukaemia, which are rarely seen in de novo AML, are frequently observed in sAML after myeloproliferative neoplasms. We report an ET with a short time of progression to a rare acute leukaemia which co-expresses markers of megakaryocytic and erythroid lineages suggesting that it's derived from progenitor cells with erythroid-megakaryocyte bipotentiality. A complex karyotype and an aggressive clinical course have been described in this kind of leukaemia.

Figure 1: immunophenotype of acute leukaemia



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Utility of flow cytometry for diagnosing a very atypical ALK+ anaplastic T-cell lymphoma

Laura Esparcia Pinedo[1], Ildefonso Sanchez Cerrillo[1], Tamara Mateu Albero[1], Ligia Gabriela Gabrie Rodriguez[1], Ana Maria Garcia-Noblejas Moya[2], Victor Lopez-Huete[1], Cecilia Muñoz Calleja[1]
[1]Immunology Service Hospital Universitario de la Princesa, Madrid, España, [2]Hematology Service Hospital Universitario de la Princesa, Madrid, España

Introduction

Anaplastic large cell lymphoma (ALCL) is a rare and aggressive T-cell non-Hodgkin lymphoma. According to the expression of the anaplastic lymphoma kinase (ALK), it is classified into ALK negative or positive ALCL, which typically affects children and young adults. ALCL is characterized by multiple lymphadenopathies and patients often suffer from B symptoms. The hemophagocytic lymphohistiocytosis is also a typical presentation.

Clinical case presentation

We present a case report of a 21-year-old man who attended the emergency with a fever of over 38ºC. The liver profile alterations and hepatomegaly suggested a mononucleosis-like syndrome. A week after, he was admitted to the intensive care unit with a severe liver failure. A CT scan revealed multiple lymphadenopathies and confirmed a massive hepatomegaly which altogether with the negative serologies suggested a lymphoproliferative syndrome and/or a hemophagocytic syndrome.

Immunophenotypic strategy and results

In light of the severity, a bone marrow sample was directly studied with 8 colour panels: an acute leukemia screening tube, an in house screening panel for either B or T lymphoma and myeloid neoplasms/myelodysplastic syndrome, and a tube for cytoplasmic perforin.
This analysis detected a pathologic cellularity with some heterogeneity in SSC/FSC parameters and phenotype. The cells were CD45+ cytCD3- CD3- CD2+/- CD7+ CD4- CD8- CD5- CD56- CD16- perforin- CD45RO+. Perforin levels were normal in the healthy T and NK cells of the patient. All B cell linage markers were negative. Antigens of immature cells (CD34, CD117, TdT, CD1a) were also absent. Surprisingly, the largest population expressed high levels of HLA-DR and CD13 whereas the smallest one showed some positivity for both antigens. The rest of the myeloid antigens, including MPO, CD15, CD11b and CD14, were negative with the exception of a weak positivity for CD33. The cytologic evaluation of the BM revealed no evidence of hemophagocytosis or hematological neoplasias.

Differential diagnoses and definitive diagnosis

With these data, the differential diagnosis between a dendritic cell neoplasm and a T/NK cell lymphoma was considered. Negativity for CD94, KIR and NKG2 molecules excluded an NK origin. Molecules identifying dendritic cells were also absent including CD1c, CD303, CD304, CD141, CD123, CD11c, CD36 and CD64. With the suspicion of a T-cell lymphoma, a detailed phenotype revealed that the pathological cells expressed CD95, CD43, CD38, CCR6 and CCR7, but were negative for CD45RA, CD279, CXCR5, CD25, and other homing and

chemokine receptors. Since CD30, which is typically expressed by ALCL, was expressed on 10% of the largest population we tried an intracellular staining of the molecule that was clearly positive.
Taking into account the whole phenotype, we proposed as the most probable diagnosis an ALCL with liver tropism, in a probable relationship with CCR6 expression.
Based on the clinical data and on our report hematologists began a specific chemotherapy for an aggressive T-cell lymphoma.
Thereafter, the liver biopsy confirmed our suspicion and the definitive diagnosis was ALK+ CD30+ ALCL.

Conclusions

This case illustrates the utility of flow cytometry even for the diagnosis of hematological neoplasms with a very atypical presentation and/or phenotype.
In addition, we have learnt the convenience of searching for certain key antigens at an intracellular level, mainly in challenging cases like this.

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Pulmonary extranodal marginal B-CELL lymphoma of mucosa associated lymph tissue (Malt) diagnosed by bronchoalveolar lavage: a case report

Maria Manuela Rebordao[1], Manuela Tata[1], Artur Paiva[2], Manuel Silva[1]
[1]Hospital Forças Armadas, Lisboa, Portugal, [2]Centro Hospitalar e Universitário de Coimbra, Coimbra, Portugal

Primary pulmonary non-Hodgkin's lymphoma (NHL) represents <1% of all NHLs and more than two-thirds of all pulmonary NHLs are Extranodal Marginal Zone B-cell Lymphoma (MZBL) of MALT type. [1It is a rare entity with a world incidence of 1-1,5 cases /10^5/year.

We report a case of 51 years old nonsmoking men referred to medical consultation because the chest radiography and CT (Computed Tomography) showed heterogeneous hypotransparency on the right upper lobe, bronchiectasis and an adjacent area of consolidation suggestive of inflammation/infection, without pleural effusion and thoracic adenopathies. The patient was asymptomatic and physical examination did not reveal any remarkable findings. Laboratory findings in peripheral blood were normal for hemogram, protein electrophoresis, Immunoglobulin assay and autoimmunity markers.The patient underwent fiberoptic bronchoscopy which did not reveal abnormal findings. Endobronchial biopsies were obtain and histologic examination showed a nonspecific inflammatory pattern. Bronchoalveolar lavage fluid (BAL) was obtained and sent for bacteriological, mycobacteriological, mycological studies which were negative.

The lymphocytic populations of BAL were studied by flow cytometry (Beckman Coulter-FS 500). We detected a clonality of proliferation B-cells CD19+ lymphocytes. This was studied with monoclonal antibodies and the results were: B cells represented 60% of all cells, with a POSITIVE phenotype for CD19, CD20, CD79, CD38, CD43, CD11c, CD95 CD81, CXCR5, CD22, CD49d, CD39 of heterogeneous expression (normal to strong) and Kappa chains. Negative Phenotype for CD23, CD10, CD5, IgM, CD103, CD27, CD62L, CD31, CD305, CD200 and Lambda chains.

Phenotyping of lymphocytes from BAL revealed the presence of two pathological B cell populationsn which differed (1) in the expression of CD95 and CD39 and (2) in their size. Thus, the phenotype suggests the presence of marginal zone B lymphoma. Besides, does not rule out the transformation into diffuse large B-cell lymphoma (larger sized B cells and CD95 +). The patient was submitted to transthoracic biopsy that confirmed the presence of Pulmonary Extranodal Marginal Zone B-cell Lymphoma of Mucosa Associated Lymph Tissue (MALT) and started chemotherapy.

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Immunophenotypic characterization of the erythroid compartment in cases of acquired pure red cell aplasia – diagnostic utility of flow cytometry

Raquel Silva[1], Yumi Maekawa[1], Tania Takao[1], Nair Tamashiro[1], Amanda Chereghini[1], Daniele Daniel[1], Ana Claudia Silva[1], Matheus Vescovi[1], Alex Sandes[1]
[1]*Fleury Group, São Paulo, Brazil*

Introduction:

The erythrovirus (parvovirus) B19 is the only Parvoviridae considered to be pathogenic to humans. It is part of the genus Erythrovirus that in contact with organism infects and destroys erythroid precursors and can cause pure red cell aplasia. Patients with this condition have anemia, decreased reticulocytes and typical morphological findings in the bone marrow aspirate, such as a marked decrease in the number of erythroblasts and the presence of giant proerythroblasts. The diagnosis is performed by PCR for virus detection or serological tests.

Methods:

We retrospectively evaluated bone marrow samples from 17 patients with bone marrow aspirate suggestive of pure red cell aplasia by parvovirus who had also performed immunophenotyping by flow cytometry. The diagnosis of parvovirus B19 infection was confirmed in 11 cases, six by molecular tests and five by the presence of IgM antibodies to B19. The remaining six cases did not test for parvovirus B19 and were excluded from the analysis. Ten normal controls were tested in parallel. To evaluate erythrocytic differentiation, a tube with the following MAbs: CD36, CD105, CD71, CD117, CD34, CD45, CD7 and CD19 was performed. The samples were acquired on the 8-color FACSCanto-II cytometer and analyzed by Infinicyt software.

Results:

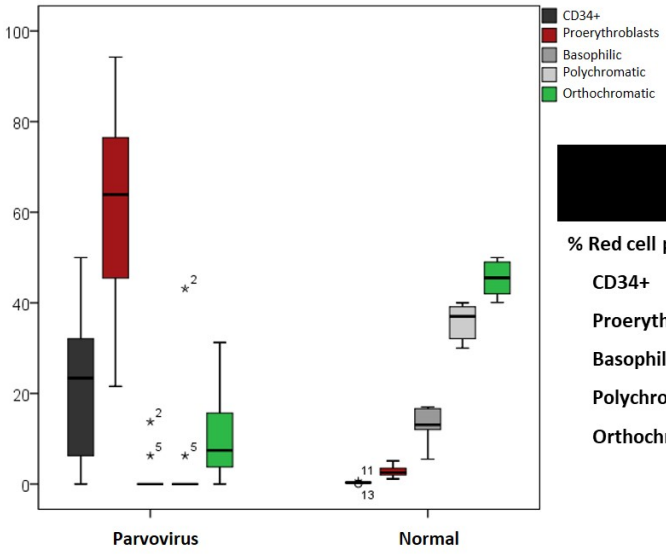
The median age of the patients was 43 years (18-68), being 6 males and 5 females. The hemogram showed Hb of 11.8 g/dL (4.9-14.1), leukocytes of $2.4x10^6$/L (1.3-6.1) and platelets of $109x10^6$/L (6-256) and all cases presented reticulocytopenia: $6,5x10^9$/L (4,6-15,7). The bone marrow aspirate showed 2% (0,8-8%) of erythroblasts and presence of giant proerythroblasts. Immunophenotyping showed intense reduction of erythroblasts, with a mean number of 0.3%±0.2. Notably, erythroblasts presented a characteristic immunophenotypic profile, with presence of cells with high light scatter properties (FSC/SSC) and important block on red cell maturation, especially on the CD34+ erythroid precursors and proerythroblasts compartments (figure 1).

Conclusions:

Flow cytometry is a diagnostic tool increasingly used in the diagnostic evaluation of unexplained anemia. Our results show that cases of acquired pure red cell aplasia present a peculiar immunophenotypic signature, characterized by an overall reduction in the number of red cell precursors and the presence of large erythroblasts (CD71+/CD36+) exclusively expressing CD34, CD117 and CD105, a profile equivalent to the morphological finding of giant proerythroblasts. We recommend performing PCR for B19 erythrovirus and/or serological tests for all cases presenting the immunophenotypic profile described, in addition to the investigation of other causative agents of erythroid aplasia.

Figure 1:

Distribution of bone marrow red cell compartments of cases with pure red cell aplasia by Parvovirus B19 and normal controls.



	Normal (mean ± s.d.)	Parvovirus B19 infection (mean ± s.d.)	*p*
% Red cell precursors	10,61 ± 4,02	0,3% ± 0,2	0,004
CD34+	0,34 ± 0,21	22,3% ± 16	0,002
Proerythroblasts	2,87 ± 1,52	61,4% ± 21	0,000
Basophilic	12,85 ± 4,65	1,9% ± 4,5	0,000
Polychromatic	35,65 ± 4,39	4,9% ± 13,9	0,000
Orthochromatic	45,32 ± 4,3	9,9 %± 9,5	0,000

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Curcumin effects in the enhancement of proapoptotic drugs on CLL cell lines

Àngel Bistué-Rovira[1], Laura Garcia[1], Jordi Juncà[1], Jorge Bardina[1], Michael Ward[2], Jolene Bradford[2], Jordi Petriz[1]

[1]Josep Carreras Leukaemia Research Institute (IJC), ICO-Hospital Germans Trias i Pujol, Universitat Autònoma de Barcelona, Badalona (Barcelona), Spain, [2]ThermoFisher Scientific, Eugene, USA

Introduction:

Chronic lymphocytic leukemia (CLL) is a hematologic disease caused by a pathological expansion of B cells due to a reduction of apoptosis. Curcumin is a natural phenolic compound with low toxicity that has demonstrated potential against different cancers, potentiating apoptotic pathways and reversing drug resistance, among other effects. The objective of this study is to determine curcumin effects on potentiation of drug toxicity, in order to decrease effective doses and reduce secondary effects.

Methods:

I83 and Mec1 CLL cell lines were incubated for 0, 24, 48 and 72 hours with different combinations of proapoptotic drugs (etoposide 0.1µM, camptothecin 0.005µM, colchicine 0.005µM, cytarabine 0.01µM and fludarabine 10µM) and curcumin (Naturex, 95% purity, used at a final concentration of 5µM). Cells were sampled and analyzed by flow cytometry using an Attune NxT flow cytometer (Thermo Fisher) for cell membrane integrity (PI staining) and cell cycle disruption (DAPI staining). Student's two tailed t-test ($P<0.05$) and FCS Express 5 Flow Research Edition (De Novo Software) were used for statistical analysis. Blood smears were stained with May Grünwald/Giemsa for microscopy analysis.

Results:

Curcumin enhanced cell membrane disruption induced by etoposide, camptothecin and colchicine in both cell lines, increasing the number of dead cells respectively by 75, 200 and 60% in I83, and by 83, 33 and 96% in Mec1 at 72 hours. It also enhanced cell proliferation blockade induced by these drugs, reducing proliferation at 72 hours by 38, 62 and 42% for I83, and by 46, 43 and 70% for Mec1, respectively. Fludarabine enhancement was higher on Mec1 than on I83, reducing cell proliferation by 33% and 14% respectively. Curcumin enhanced S phase cell cycle arrest produced by cytarabine and fludarabine on both cell lines, produced cytoplasm vesicle formation in I83 when combined with etoposide, and accelerated cytarabine and fludarabine induced apoptosis from 48 to 24 hours in Mec1.

Conclusions:

Curcumin enhanced the effects of all tested drugs, with variations depending on drug and cell line. Curcumin strongly potentiated colchicine and camptothecin effects on cell membrane disruption and proliferation blockage. It is worth noting that these drugs are effective against cancer but have toxicity limitations. Curcumin had higher effect potentiating fludarabine on Mec1 than on I83. Interestingly, Mec1 are known to have mutated *TP53*, which confers chemoresistance to fludarabine, while curcumin effects regulating the expression of this gene have been reported, presenting as a possibility to partially reverse chemoresistance to this drug.

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Phenotypical and functional alterations of different peripheral blood populations of monocytes and dendritic cells in early psychosis

Andreia Sofia Diniz[1], Nuno Madeira[2], Manuel Coroa[2], Sofia Morais[2], Cátia Santa[3], Isabel Silva[1], Bruno Manadas[3], António Macedo[2], Artur Paiva[1]

[1]Operational Management Flow Cytometry Unit/Clinical Pathology Service, Coimbra Hospital and University Centre, Coimbra, Portugal, [2]Psychiatry Department, Coimbra Hospital and University Centre, Coimbra, Coimbra, Portugal, [3]CNC - Center for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal

Studies in the last two decades identified a complex interaction between immune system, systemic inflammation and the brain, leading to humour, cognition and behaviour dysfunctions. While the evidence of changes in immune cells and cytokine production in the peripheral immune system of schizophrenia becomes clear, the mechanisms involved in the central nervous system (CNS) and peripheral regulation need to be elucidated. Although most studies rely on pathological mechanisms acting on the CNS, such neuroinflammation could act on peripheral inflammatory systems, activating the immune system. Monocytes and dendritic cells are key cells in innate immunity, having crucial function in activation of immune system. These antingen presenting cells are versatile, regulate the inflammatory process and induce immunity. Our goal was to understand if these populations are altered in schizophrenia, becoming a potential target of monitorization the course of this psiquiatric disease.

In this study, we addressed monocytes subpopulations: classical monocytes, intermediate monocytes, non-classical monocytes and monocytes expressing IgE binding receptor (FcεRI); and dendritic cells (plasmacytoid and myeloid) in early psychosis. First we focused on the differences between First-Episode Psychosis (FEP) patients (N=27, mean age 26, 21 males and 6 female) and healthy volunteers (N=10, mean age 30, 7 males and 3 female) with regard to peripheral blood monocytes subpopulations and dendritic cells, and later re-evaluating patients after short-term antipsychotic treatment, early psychosis patients (N=8; mean age 30, 6 males and 2 female). With this in mind, we proceeded with quantification of monocytes subpopulations and myeloid dendritic cells producing intracellular cytokines (TNF and IL-6) with and without a stimulation with LPS (Lipopolysaccharide) and IFN-γ, by flow cytometry.

In drug-naïve FEP patients our results showed an increased frequency of non-classical monocytes producing IL-6, and increased production of this cytokine per cell in all subpopulations studied, except plasmacytoid dendritic cells. Patients with FEP in early treatment had an increase of cell's frequency producing TNF-α which, in association with increased HLA-DR expression, suggests an inflammatory cell profile. The most interesting

finding was an increased expression of IgE binding its receptor (FcεRI), indicating increased serum IgE in FEP patients.

Taken together, our findings demonstrate phenotypical and functional alterations in monocytes in early psychosis, namely non-classical monocytes, and increased IgE binding in their receptors, suggesting such findings as potential biomarking targets in early phases of psychosis.

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Functional and phenotypic characterization of tumour-infiltrating leukocytes in human tumour biopsies from hepatocellular carcinoma and cholangiocarcinoma patients

Carmen Martín-Sierra[1,2], Ricardo Martins[2,3,4,5], Paula Laranjeira[1,2], Ana Margarida Abrantes[2,5], Rui Caetano Oliveira[2,5,6], José Guilherme Tralhão[2,3,4,5], Maria Filomena Botelho[2,5], Emanuel Furtado[3], Rosario Domingues[7], Artur Paiva[1,2,8]

[1]Unidade de Gestão Operacional em Citometria, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal, [2]Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra, Coimbra, Portugal, [3]Unidade Transplantação Hepática Pediátrica e de Adultos, Centro Hospitalar e Universitário de Coimbra (UTHPA, CHUC), Coimbra, Portugal, [4]Serviço de Cirurgia Geral, Unidade HBP, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal, [5]Instituto de Biofísica, IBILI, Faculdade de Medicina, Universidade de Coimbra, Coimbra, Portugal, [6]Serviço de Anatomia Patológica, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal, [7]Mass Spectrometry Centre, Department of Chemistry & QOPNA, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal, [8]Instituto Politécnico de Coimbra, ESTESC-Coimbra Health School, Ciências Biomédicas Laboratoriais, Coimbra, Portugal

Introduction:

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) represent the most common primary liver malignancies whose outcome is influenced by the immune response. The purpose of this work is to identify the tumour-infiltrating leukocyte (TIL) subsets, and their distinct functions in the tumour niche, in these two groups of cancer patients and to determine the differences associated to the different tumour grades or stages (TNM).

Methods:

We have characterized, by flow cytometry, the TIL populations of a group of patients with HCC (n=20) and a group of patients with CCA (n=8), at the time point of the surgical resection. Moreover, $CD4^+$ T cells, $CD8^+$ T cells and antigen-presenting cells were purified by cell sorting for further analysis of gene expression by qPCR.

Results:

Regarding tumour infiltrating macrophages phenotype, we have observed a significantly higher expression of markers associated with M2 phenotype (CD206 and CD163) and a higher expression of PD-L1 (CD274) in patients with HCC in comparison to CCA patients. In addition, for HCC patients, we have observed a significant increase in the expression of CD200R in macrophages from tumours that were in grade G3 or G4, in comparison to tumours in grade G1 or G2. Macrophages from HCC biopsies presented a higher expression

of IL-10 mRNA than CCA macrophages. Interestingly, we found a positive correlation between CD163 protein expression (measured as mean fluorescence intensity, MFI) and IL-10 mRNA expression, for HCC-infiltrating macrophages. Regarding tumour infiltrating lymphocytes, we have observed a significantly higher frequency of tumour infiltrating lymphocytes, $CD8^{+}CD56^{+}$ T cells and NK in HCC biopsies in comparison to CCA. Additionally, a consistent decrease in IFNγ MFI associated with advanced TNM stages was observed in all T cell subsets present in HCC biopsies; likewise, a significant decrease in the frequency of tumour-infiltrating Th17 and Tc17 cells was associated with advanced stages of HCC tumours.

Conclusions:

Our results indicate a higher infiltration of M2 macrophages in HCC biopsies, in comparison to CCA, a feature that has been associated with a worst prognosis. Moreover, our results point to a higher protein expression levels of CD200R in macrophages infiltrating high-grade tumours (G3 and G4) in HCC patients. In both groups of cancer patients, a negative correlation between IFNγ MFI and TNM stages was observed suggesting an important role for IFNγ producing cells in cancer progression.

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Functional impairment of circulating precursors (FcεRI+ monocytes) and fully differentiated myeloid dendritic cells in hepatocellular carcinoma and cholangiocarcinoma patients

Carmen Martín-Sierra[1,2], Ricardo Martins[2,3,4,5], Paula Laranjeira[1,2], Ana Margarida Abrantes[2,5], Rui Caetano Oliveira[2,5,6], José Guilherme Tralhão[2,3,4,5], Maria Filomena Botelho[2,5], Emanuel Furtado[3], Rosario Domingues[7], Artur Paiva[1,2,8]

[1]Unidade de Gestão Operacional em Citometria, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal, [2]Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculty of Medicine, University of Coimbra, Coimbra, Portugal, [3]Unidade Transplantação Hepática Pediátrica e de Adultos, Centro Hospitalar e Universitário de Coimbra (UTHPA, CHUC), Coimbra, Portugal, [4]Serviço de Cirurgia Geral, Unidade HBP, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal, [5]Instituto de Biofísica, IBILI, Faculdade de Medicina, Universidade de Coimbra, Coimbra, Portugal, [6]Serviço de Anatomia Patológica, Centro Hospitalar e Universitário de Coimbra (CHUC), Coimbra, Portugal, [7]Mass Spectrometry Centre, Department of Chemistry & QOPNA, University of Aveiro, Campus Universitário de Santiago, Aveiro, Portugal, [8]Instituto Politécnico de Coimbra, ESTESC-Coimbra Health School, Ciências Biomédicas Laboratoriais, Coimbra, Portugal

Introduction:

Hepatocellular carcinoma (HCC) and cholangiocarcinoma (CCA) represent the most common primary liver malignancies whose outcome is influenced by the immune response. Tumour immune evasion has been previously associated with the defective dendritic cells (DC) function in cancer patients as a result of decreased numbers of competent DC and accumulation of immature cells. Therefore, in this study, we aimed to identify and characterize circulating myeloid dendritic cells (mDCs) and FcεRI$^+$ monocytes in peripheral blood from HCC and CCA patients.

Methods:

In this study, we have functionally characterized, by flow cytometry, circulating mDCs and FcεRI$^+$ monocytes in a group of healthy individuals (n=10) and in a group of patients with HCC (n=19) and CCA (n=8), at the time point of the surgical resection (T0) and once the patient had recovered from surgery (T1). Moreover, we proceeded to a more in-depth phenotypic characterization of the FcεRI$^+$ monocyte subpopulation.

Results:

A significant decrease in the frequency of TNFα producing FcεRI$^+$ monocytes and mDCs in HCC and CCA patients when compared to the group of healthy individuals was observed, and a close association between FcεRI$^+$ monocytes and mDCs dysfunction was identified. In

addition, the phenotypic characteristics of FcεRI$^+$ monocytes from healthy individuals strongly suggest that this population drives to mDCs, which matches with the fact that both populations are functionally affected.

Conclusions:

The frequency and the function of circulating mDCs and FcεRI$^+$ monocytes are affected in both HCC and CCA patients and FcεRI$^+$ monocytes could represent those fated to become mDCs.

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Early recovery of naïve T cells predicts improved survival after allogeneic hematopoietic stem cell transplantation

Itxaso Portero Sainz[1], Yaiza Perez Garcia[1], Valle Gómez Garcia de Soria[2], Victor López Huete[1], Ana Ramirez Mengíbar[1], Cecilia Muñoz Calleja[1]

[1]Servicio de Inmunología e Instituto de Investigación Sanitaria del Hospital Universitario de la Princesa, Madrid, Spain, [2]Servicio de Hematología e Instituto de Investigación Sanitaria del Hospital Universitario de la Princesa, Madrid, Madrid, Spain

Introduction:

Allogeneic stem cell transplantation (alo-HSCT) is a curative treatment but the pattern and quality of the immune reconstitution (IR) after transplantation may affect its outcomes. Since there are limited data regarding the IR, the objective of this study is to investigate the association of the IR and its effects on the graft *versus* host disease (GVHD) and survival.

Methods:

Eighty-five patients who received a non-T-cell depleted alo-HSCT in our center from 2011 to 2014 were prospectively studied. Lymphocyte immunophenotyping was performed by flow cytometry at days +30, +60, +90, +180 and +360 after alo-HSCT to quantify total CD4+ and CD8+ T cells, both naïve and central memory T lymphocytes. The association between IR and the GVHD was studied through an ANOVA. For the multivariate analysis, a logistic regression was performed including those confusing clinical variables that were significant in the univariate analysis ($p \leq 0.10$). The study of overall survival (OS) *versus* IR was performed with a cox regression model.

Results:

The absolute counts of CD3+ T lymphocytes reached normal numbers within the first two months and CD8+ T lymphocytes recovered much faster than T CD4+. Conversely, it took nearly one year to get normal counts of CD4+ T cells, resulting in the expected perturbation of the CD4/CD8 ratio.
For their part, the only two clinical parameters conditioning a worse recovery of the CD4+ T cells were the previous alosensitization of the donor and the sex, being female donor and male recipient the worst combination for the IR. No parameters influenced the quality of the reconstitution of CD8+ T cells. Of note, the age or the HLA status did not influence the quality of the IR.
Interestingly, when the patients were divided into GVHD and no GVHD, we found no differences in the recovery of every T cell subpopulation, including total T cells as well as naïve/central memory T cells, both CD4+ and CD8+.

Finally, a multivariant analysis confirmed that the absolute counts of CD4+CCR7+ T cells at day +90 as well as the absolute counts of both CD4+CCR7+ T cells and naïve CD4+CCR7+CD62L+ at day +180 were associated with better OS.

Conclusions:

In conclusion, neither the development of GVHD nor other parameters seem to play a determinant role in the quality of the IR.

Of note, to our knowledge this is the first study which demonstrate an association between the recovery of naïve CD4+ T cells and the OS.

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Decreased peripheral dendritic cells (DCs) in decompensated cirrhosis. Flow cytometric detection of DCs and its relation to disease severity

Chandra Chiappin Cardoso[1], Carolina JH Pereira[1], Camila Matiollo[1], Janaina S Fonseca[1], Helder EL Alves[1], Otavio M Silva[1], Caudia R Dos Santos[1], Leonardo L Schiavon[1], Maria Claudia Santos-Silva[1]
[1]*Universidade Federal de Santa Catarina, Florianopolis, Brazil*

Introduction:

Cirrhosis occurs in response to chronic liver injury. During disease course, the immune system is deeply affected, which results in immune dysfunction associated with cirrhosis. Dendritic cells (DCs) play an important role in innate and adaptive immunity. Previous authors described cellular disturbances in cirrhotic patients (CP), as reduction of T and B cells subsets; however, so far, DCs frequencies and its relation to disease severity have not been evaluated. Thus, this study aims to evaluate the frequency and absolute numbers of circulating DCs subsets (myeloid – mDCs and plasmacytoid DCs – pDCs) in CP.

Methods:

This study included 40 CP hospitalized for decompensated cirrhosis at University Hospital of Santa Catarina, Brazil. The healthy control group (HC) included 30 age and gender matched individuals. Written informed consent was obtained from all participants. The study was approved by the ethics committee of the University. EDTA-peripheral blood (PB) samples were collected prospectively. For DCs analysis, 300 uL of PB were used for staining of the following antibody panel: HLA-DR PacB/CD45PacO/CD16FITC/CD123PE/CD11cPerCPCy5.5/CD10PC7/CD14APC/Lin(CD3,CD19 ,CD20)APCH7. Data acquisition was performed using a FACSCanto II cytometer (BD, USA). In order to detect DCs subsets, 500.000 to one million gated $CD45^+$ events were recorded per tube. Data analysis was performed using Infinicyt software version 1.7.0 (Cytognos, Spain). For absolute numbers calculation, samples were processed by the equipment XE 2100 (Sysmex, Japan). Data were analyzed using IBM SPSS Statistic software and *P* value was considered significant when <0.05.

Results:

Total DCs, mDCs, CD16+ mDCs, and pDCs are decreased in CP compared to HC ($p<0.01$). In addition, considering only DCs compartment, CP showed lower levels of mDCs and higher levels of pDCs than HC ($p<0.05$), these results were highlighted when patients with Child-Pugh B or C (worst prognosis) were separated ($p<0.05$). The higher the CP´s MELD scores

(worst prognosis) the lower the frequencies of total DCs, mDCs, and pDCs (p<0.05). Child-Pugh C-CP also presented lower frequencies of total DCs, mDCs and CD16+ mDCs than Child-Pugh B-CP (p<0.05). Additionally, DCs counts were negatively correlated with CRP (p<0.01) and bilirubin (p<0.05) levels, and positively with platelets counts (p<0.01). Concerning disease ethiology (alcoholic or viral), there were no statistical differences.

Conclusion(s):

These results demonstrated that circulating DCs numbers are reduced in CP and that this reduction associates with disease severity. Further studies including CP with stable disease are needed to determine the usefulness of DCs subsets monitoring as such a biomarker of disease progression.

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Assessment of oxaliplatinum-desensitizacion treatment by basophil activation test

María Gutiérrez-Larrañaga[1], Leticia De las Vecillas[1], Sandra Guiral[1], Laura Riesco[1], J Casanova-Vázquez[1], R. De la Puerta-Huertas[1], E. Martínez de Castro[1], Fernando Rodríguez-Fernández[1], Marcos López-Hoyos[1], David San Segundo[1]

[1]*Hospital Universitario Marqués De Valdecilla-IDIVAL, Santander, Spain*

Introduction:

The oxaliplatin (OX) is a 3rd generation platinum compound used as first line therapy in colorectal cancer, breast, non-small cell lung, ovarian, prostate and stomach cancer. The incidence of hypersensitivity reactions with OX is estimated in more than 20%, between the 7º and 10º life time exposure[1,2]. Drug desensitization (DS) provides a useful and safe tool to maintain patients on their best therapy option after a severe hypersensitivity reaction. The aim of this work was to evaluate the basophil activation test (BAT) to OX as a tool to identify potential patients at risk of suffering breakthrough reactions during DS and the *in vitro* effect of DS.

Methods:

Six patients with confirmed hypersensitivity infusion reaction to OX (positive skin test) in our institution were recruited after consent given. The BAT was performed pre- and post-DS treatment (1st, 3rd and 5th DS). The OX concentrations were used as previously described[3]. At least 20 basophils were acquired by flow cytometry and identified as SSC^{lo} FSC^{lo} $CD123^{+}$ HLA-DR^{-} $CD203c^{+}$. A BAT positive result was considered when [(maximum $\%CD63^{+}$ on basophils) – ($\%CD63^{+}$ basophils resting)] > 4.7% (BAT quantitative result).

Results:

Two out of 6 patients with OX-ADR, have a positive BAT result in the first pre-DS sample and only one patient remained positive post-DS. No reaction was observed during any DS infusion. After DS treatment a significant decrease in basophil´s recovery was observed (p=0.0012; Figure 1A) so some pre-post DS BAT were not interpretable. In those patients with enough basophil events to analyze (pre-post DS, n=13), the mean BAT quantitative result was (-0.83+/-12.3%). Individually, after increasing the number of DS, the basophil activation was reduced (Figure 1B).

Conclusion(s):

In our modest cohort, an initial positive BAT result did not predict any reaction during DS treatment. Monitoring quantitative BAT result pre-post DS confirmed *in vitro* the effectiveness of DS treatment already shown *in vivo* with a decrease basophil number and activation.

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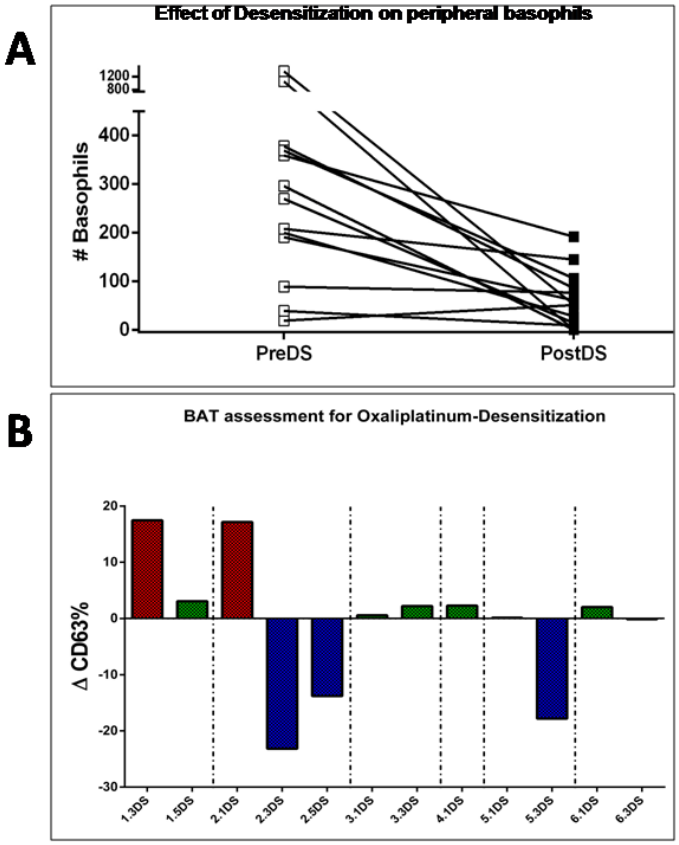
Figure 1. BAT assessment of Desensitization treatment to Oxiplatinum.



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High Quality Multiparametric Flow Cytometry: Seeing the Full Picture Through Full Spectrum Cytometry

Yacine Kharraz[1], Huimin Gu[1], Alex Zhong[1], Maria Jaimes[1]
[1]*Cytek Biosciences, Fremont, United States*

Introduction:

Full Spectrum Cytometry differs from conventional cytometry in the way that light emitted from the excitation of a particular fluorochrome is collected. Rather than collect this light at only the peak emission wavelength, full spectrum cytometry captures this light across a range of wavelengths from UV to far red (420-830 nm). This strategy makes it possible to approach multicolor flow cytometry in a more flexible way when it comes to fluorochrome choices and enables high dimension (> 20 color) high resolution flow assays with fewer lasers. The aim of this work was to optimize a 24-color panel for immunophenotyping in human whole blood.

Methods:

An assay aimed at identifying the main circulating cell subsets in whole blood was designed and included 24 different markers. 53 commercially available fluorochromes were characterized in terms of spectrum signature, brightness and spread using an Aurora full spectrum cytometer equipped with 3 lasers (405, 488 and 635 nm). 24 fluorochromes that could be used in combination on the 3 laser Aurora were selected. Following the principles of panel design, a theoretical panel was created. Whole blood from normal donors was used to evaluate the panel performance, and both bead and cell controls were tested to identify optimal controls for best panel performance.

Results:

Detailed analysis of the single stained controls revealed that beads were not always optimal controls as the spectral characteristics of these controls sometimes differed from cell controls. Moreover, the analysis of the initial multicolor panel showed good resolution for the great majority of the markers but needed further optimization to better resolve 3 out of the 24 markers. A second panel was designed and showed optimal resolution of all the markers in the panel. Manual and automated analysis of the data showed that all populations of interest were clearly identified.

Conclusion(s):

Developing a highly multiparametric panel using a full spectrum flow cytometer proved to be a straightforward process that resulted in high resolution data. The possibility to fully assess the spectrum of each dye not only guided fluorochrome selection but was also key for successful full spectrum cytometry panel design.

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Effectiveness and safety assessment of biological-drug desensitizacion treatment by basophil activation test

María Gutiérrez-Larrañaga[1], Leticia De las Vecillas[1], Sandra Guiral[1], Laura Riesco[1], A. García de la Paz[1], S. González-Barrera[1], A García-Castaño[1], M. Gutiérrez-Martínez[1], Fernando Rodríguez-Fernández[1], Marcos López-Hoyos[1], David San Segundo[1]

[1]*Hospital Universitario Marqués De Valdecilla-IDIVAL, Santander, Spain*

Introduction:

The sensitivity of actual in vitro test to identify drug-hypersensitivity is low. The basophil activation test (BAT) allow the optimization and conducting with any allergen in soluble phase. The BAT is gaining relevance as complimentary test in the study of adverse-drug reactions (ADR). The increase use of monoclonal antibodies in onco-hematologic pathology has lead an increase in hypersensitivity reactions. By re-introducing the treatment in desensitization (DS) regimen, the patient is allowed to receive his best therapeutic option protecting him from suffering an anaphylactic reaction. The confirmation of hypersensitivity by in vivo test (cutaneous test) shows a limited sensitivity (40-50%)[1]. Recent works point to BAT as a safe tool for the in vitro study of monoclonal antibodies hypersensitivity as rituximab (RTX)[2] and cetuximab (CTX)[3]. The aim of the present work was to assess the BAT utility in ADR with monoclonal antibodies.

Methods:

Two patients with oncologic pathology that suffered anaphylactic reactions after RTX and CTX infusion were selected. Both patients had positive cutaneous test results. The BAT was set up with drug concentration as previously used[2,3] and performed pre- and post-DS. A minimum of 50 basophils were identified as SSC^{lo} FSC^{lo} $CD123^{+}$ HLA-DR^{-} $CD203c^{+}$ and the activation was defined by expression of CD63 (positive >5%). The performance of the DS treatment was assessed as 63: [(%CD63 post-DS) - (%CD63 pre-DS)].

Results:

Both patients had positive BAT results in each pre-DS treatment, and a mild reaction during the first-DS infusion was observed. The patient with RTX-hypersensitivity had another positive BAT result pre-second-DS, however no reaction during infusion was detected, the 63 was = -27.6% (with negative BAT result after DS). The patient with CTX-hypersensitivity had positive BAT results pre 2nd and 3rd DS (5% and 35.1%, respectively) and was treated with anti-histamine and steroids during infusion without ADR, however no performance of DS was tested.

Conclusion:

TAB has shown its usefulness to confirm in vitro hypersensitivity to monoclonal antibodies, but also its result could have relevance as a safety marker of the DS guidelines and as a predictor of adverse reactions during DS.

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T-cell Subpopulations in Patients with Idic15 Syndrome, a Rare Disease of the Autistic Spectrum

Beatriz Jávega[1], Guadalupe Herrera[2], Sandra Tavárez[2], Macarena Izquierdo-Peyrallo[2], Miguel-Angel García[4], Pilar Codoñer[5], Miguel-Angel Orquín[6], Eulalia Alonso[2], José-Enrique O'Connor[1]

[1]Laboratory of Cytomics, Joint Research Unit CIPF-UVEG, Valencia, Spain, [2]Servicio de Citometría, UCIM, IIS INCLIVA -Universidad de Valencia, Valencia, Spain, [3]Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, Valencia, Spain, [4]Departamento de Genetica e IIS INCLIVA, Universidad de Valencia, Valencia, Spain, [5]Departamento de Pediatría, Obstetricia y Ginecología-Servicio de Pediatría, Hospital Universitario Doctor Peset, Valencia, Spain, [6]Servicio de Medicina Familiar y Comunitaria, Centro de Salud de Benicarló y Asociación Idic15, Benicarló, Spain

Introduction:

The Idic15 syndrome is a rare neurological disease caused by duplications of the 15q11-q13 region, in which some genes related to the immune response are found. A study in progress on young patients diagnosed with Idic15 has shown a high incidence of recurrent infections. Therefore, the possible relationship between 15q11-13 duplications and changes in the cellular immune functions has been addressed through an immunophenotypic study of T-cell subpopulations by polychromatic flow cytometry.

Methods:

Study included 28 young patients (3-19 years) diagnosed with Idic15, recruited among the members of the Idic15 Spain Association, and 17 controls matched by age, sex and geographical area. EDTA-anticoagulated whole-blood samples were immediately stabilized with Transfix (Cytomark) and analyzed prior to 24 hours with a Gallios flow cytometer (Beckman-Coulter) using two separate commercial pre-designed 8- or 9-color panels of monoclonal antibodies (Beckman-Coulter): DuraClone IM Phenotyping Basic Tube (CD16-FITC, CD56-PE, CD14-PC7, CD4-APC, CD8-AF700, CD3-APC·AF750, CD45-KO) and DuraClone IM T cells (CD45RA-FITC, CD197-PE, CD28-ECD, CD279-PC5.5, CD27-PC7, CD4-APC, CD8-AF700, CD3-APC·AF750, CD57-PB, CD45 KO). Raw flow cytometric data were analyzed off-line with Kaluza software (Beckman-Coulter) and correlated in patients with the presence of autism and the type of duplication, according to the aCGH of the breakpoints (BP) described in the 15q11-q13 region (BP1-BP5, BP1-BP3, BP1-BP2 and BP2 -BP3).

Results:

Our results show that patients with Idic15 exhibited changes in some T-cell subpopulations with respect to controls, as well as variations related to the presence or absence of autism

and the type of rupture. Most important modifications in patients were increased numbers of Naïve CD8 T-cells and of Central Memory (bot CD4 and CD8 T-cells), and decreased numbers of Naïve CD4 T-cells and of Effector Memory CD8 T-cells, with some qualitative variations according to the type of chromosomal lesion.

Conclusions:

These results show alterations in the abundance of specific T-cell differentiated subpopulations, suggesting possible issues in immune regulation in these patients, which might be related to the increased rate of infections in Idic15. Although these results are consistent with the observations described in the general population with autism, they represent the first specific study in patients with idic 15. Project funded by donations to the "Una casa, una vida" Initiative promoted by Great Chance SLU.

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B-cell Subpopulations in Patients with Idic15 Syndrome, a Rare Disease of the Autistic Spectrum

Beatriz Jávega[1], Guadalupe Herrera[2], Sandra Tavárez[3], Macarena Izquierdo-Peyrallo[3], Miguel-Angel García[4], Pilar Codoñer[5], Miguel-Angel Orquín[6], Eulalia Alonso[3], José-Enrique O'Connor[1]

[1]Laboratory of Cytomics, Joint Research Unit CIPF-UVEG, Valencia, Spain, [2]Servicio de Citometría, UCIM, IIS INCLIVA -Universidad de Valencia, Valencia, Spain, [3]Departamento de Bioquímica y Biología Molecular, Universidad de Valencia, , Spain, [4]Departamento de Genetica e IIS INCLIVA, Universidad de Valencia, Valencia, Spain, [5]Departamento de Pediatría, Obstetricia y Ginecología-Servicio de Pediatría, Hospital Universitario Doctor Peset, Valencia, Spain, [6]Servicio de Medicina Familiar y Comunitaria, Centro de Salud de Benicarló y Asociación Idic15, Benicarló, Spain

Introduction:

The Idic15 syndrome is caused by duplications of the 15q11-q13 region, in which several immune-response genes are found. A current study on young patients diagnosed with Idic15 has shown a high incidence of recurrent infections. Therefore, we have investigated the possible relationship between 15q11-13 duplications and changes in the humoral immune responses by means of an immunophenotypic study of B-cell subpopulations by polychromatic flow cytometry.

Methods:

We have studied 28 young patients (3-19 years) diagnosed with Idic15, recruited among the members of the Idic15 Spain Association, and 17 controls matched by age, sex and geographical area. EDTA-anticoagulated whole-blood samples were immediately stabilized with Transfix (Cytomark) and analyzed prior to 24 hours with a Gallios flow cytometer (Beckman-Coulter) using two separate commercial pre-designed 8-color panels of monoclonal antibodies (Beckman-Coulter): DuraClone IM Phenotyping Basic Tube (CD16-FITC, CD56-PE, CD14-PC7, CD4-APC, CD8-AF700, CD3-APC·AF750, CD45-KO) and DuraClone IM B cells (Anti-sIgD-FITC, CD21-PE, CD19-ECD, CD27-PC7, CD24-APC, CD38-AF700, Anti-sIgM-PB, CD45 KO). Raw flow cytometric data were analyzed off-line with Kaluza software (Beckman-Coulter) and correlated in patients with the presence of autism and the type of duplication, according to the aCGH of the breakpoints (BP) described in the 15q11-q13 region (BP1-BP5, BP1-BP3, BP1-BP2 and BP2 -BP3).

Results:

Our results show that patients with idic15 had slightly decreased number of circulating B cells, especially in patients without autistic traits. In Idic15 patients without autism we found also increased Naïve B-cells and Plasmablasts, but decreased numbers of Class-Unswitched Memory B-cells. The correlation of such changes with the degree of chromosomal abnormality is less clear and requires further analysis.

Conclusions:

Our results show alterations in the abundance of specific B-cell differentiated subpopulations, suggesting possible impairment of immune effector mechanisms in Idic15 patients, which might be related to their increased susceptibility to infections. These results are also consistent with the observations described in neurological patients and represent the first specific immunological study in patients with Idic 15. Project funded by donations to the "Una casa, una vida" Initiative promoted by Great Chance SLU.

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Flow Cytometric Multiplexed Analysis of Circulating Adipokines in Young Patients with Idic15 Syndrome, a Rare Disease of the Autistic Spectrum

Beatriz Jávega[1], Macarena Izquierdo-Peyrallo[2], Alicia Martínez-Romero[1], Guadalupe Herrera[3], Sandra Tavárez[2], Miguel-Angel García[4], Pilar Codoñer[5], Miguel-Angel Orquín[6], Eulalia Alonso[2], José-Enrique O'Connor[1]

[1]Laboratory of Cytomics, Joint Research Unit CIPF-UVEG, Valencia, Spain, [2]Departamento de Bioquímica y Biología Molecular, Universidad de Valencia , Valencia, Spain, [3]Servicio de Citometría, UCIM, IIS INCLIVA - Universidad de Valencia, Valencia, Spain, [4]Departamento de Genetica e IIS INCLIVA, Universidad de Valencia, Valencia, Spain, [5]Departamento de Pediatría, Obstetricia y Ginecología-Servicio de Pediatría, Hospital Universitario Doctor Peset, Valencia, Spain, [6]Servicio de Medicina Familiar y Comunitaria, Centro de Salud de Benicarló y Asociación Idic15, Benicarló, Spain

Introduction:

The Idic15 syndrome is caused by duplications of the 15q11-q13 region and patients display severe neurological disorders, which may include autism. Association of neurological disorders of the autistic spectrum with chronic inflammation and insulin resistance has been previously reported. Adipokines are cytokines released by adipocytes that have been proposed as biomarkers of inflammation and metabolic alterations in many situations, including neurological diseases. Because of that, we have investigated the possible relationship between 15q11-13 duplications and circulating adipokines by means of a flow cytometric multiplexed assay.

Methods:

We have studied 28 young patients (3-19 years) diagnosed with Idic15, recruited among the members of the Idic15 Spain Association, and 17 controls matched by age, sex and geographical area. The levels of four main adipokines, namely adiponectin, adipsin, leptin and resistin were determined in serum samples by a bead-based multiplexed assay (LegendPlex Human Metabolic Panel 1, BioLegend) run on a CytoFlex flow cytometer (Beckman Coulter) and duplicated on a Fortessa flow cytometer (Becton Dickinson). Raw flow cytometric data were analyzed off-line with LegendPlex software (BioLegend) and correlated in patients with the presence of autism and the type of duplication, according to the aCGH of the breakpoints (BP) described in the 15q11-q13 region (BP1-BP5, BP1-BP3, BP1-BP2 and BP2 -BP3).

Results:

Our results show that patients with idic15 have a marked decrease in the levels of leptin and minor reductions in the levels of adipsin and resistin. These changes were more evident in patients of the BP1-BP2 and BP2-BP3 subgroups. On the contrary, adiponectin is slghtly increased in patients, especially in the subgroups BP1-BP3 and BP1-BP5. The changes observed were less evident in patients showing autistic manifestations.

Conclusions:

Our results show complex changes in the pattern of circulating adipokines in Idic15 patients that suggest a latent pro-inflammatory condition. While these changes seem correlated to the type of chromosomal lession, there is no positive correlation with the presence of autistic traits. In general, our results are consistent with the observations described in several neurological conditions and represent the first study of this type in patients with Idic 15. Project funded by donations to the "Una casa, una vida" Initiative promoted by Great Chance SLU.

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Effect of genetic selection and vitrification on immunological parameters after Staphylococcus aureus infection

Elena Moreno-Grua[1], Sara Pérez-Fuentes[1], Asunción Muñoz-Silvestre[1], David Viana[1], Laura Selva[1], Alberto Arnau-Bonachera[1], Eugenio Martínez-Paredes[2], Pablo Marín-García[2], Juan José Pascual[2], Juan Manuel Corpa[1]

[1]Universidad CEU Cardenal Herrera, Valencia, Spain, [2]Universitat Politècnica de València, Valencia, Spain

Introduction:
The use of immunological methods has already proven very useful when comparing different groups of rabbits subjected to various challenges. Vitrification is an embryo-freezing technique that allows animals of several generations to be available at the same time. A comparison of distinct white blood cell populations of commercial rabbits from a parental line separated by 18 generations (vitrified and non-vitrified) was made herein. The objective was to know if selection in commercial rabbits by productive characters and vitrification would affect their immune system after a *Staphylococcus aureus* infection.

Methods:
The study animals were 92 reproductive female rabbits of parental line R, which were divided into three groups: (1) current non-vitrified generation (n=24); (2) current vitrified generation (n=31); (3) vitrified group with 18 prior generations to the current generation (n=37). All the animals were intradermally-inoculated in their back with 300 colony-forming units of an *S. aureus* strain. Blood samples in EDTA anticoagulant were obtained from the median artery of the ear at 0, 1, 3 and 7 days post-infection. A flow cytometric analysis of white blood cells (B, T, T CD4+, T CD8+ and activated T-cells, monocytes and granulocytes) was performed.

Results:
Differences were observed between the current vitrified generation group and the 18 generations prior to the current generation group. Haemoglobin level and red blood cells counts were higher in the current vitrified generation group. Total lymphocytes, B lymphocytes, CD8 lymphocytes and CD25 cells were higher in the 18 generations prior to the current generation group. No differences in the number of infected animals were observed among the studied groups at 7 days after inoculation.

Conclusions:
The selection process by productive characters that occurred in the parental line seemed to negatively affect some immune parameters, but the percentage of infected animals in the current vitrified generation group was no higher than the group of 18 generations prior to the current generation group.

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Two panels to analysis the cellular composition of glioblastoma tumors using multiparametric flow cytometry

María González-Tablas Pimenta[1,2], Álvaro Otero Rodriguez[1,3], Pablo Sousa Casanovas[1,3], Daniel Ángel Arandia Guzmán[1,3], Laura Ruíz Martín[1,3], Daniel Pascual Argente[1,3], Andoni García[1,3], J. Alberto Orfao de Matos[1,2,4], Mª Dolores Tabernero Redondo[1,2,4,5]

[1]Instituto de Investigación biomédica de Salamanca, IBSAL- University Hospital of Salamanca,, Salamanca, Spain, [2]Centre for Cancer Research (CIC-IBMCC; CSIC/USAL; IBSAL) and Department of Medicine, University of Salamanca, Salamanca, Spain, [3]Neurosurgery Service of the University Hospital of Salamanca, Salamanca, Spain, [4]Biomedical Research Networking Centre on Cancer–CIBER-CIBERONC, Institute of Health Carlos III, Madrid, Spain, [5]Instituto de Estudios de Ciencias de la Salud de Castilla y León (IECSCYL-IBSAL), Salamanca, Spain

Introduction:

Glioblastoma multiforme (GBM) is the most common and aggressive primary adult brain tumor. These tumors display a high degree of cellular heterogeneity including a complex component of immune cells that contribute to tumor microenvironment.

Methods:

Two different panels were designed to characterize the cellular composition of a total of 35 GBM tumors by multiparametric flow cytometry (MFC) technique. Firstly, 10 GBM tumors were stained with a 5-color combination panel (Panel *1 Canto)* to detect antigen presenting cells (APCs) and lymphoid infiltration in FACSCanto™ cytometer (BD Biosciences). Secondly, 25 GBM tumors, stained with an 8 combination of antibodies (*Panel 2 Fortessa*) were acquired in a LSI FortessaX20 System (BD Biosciences) in order to deeply characterize myeloid cells, as well as neutrophils and microglia. The tumor fraction was assessed according to their positivity for GFAP astrocytic marker, adhesion markers (CD24, CD44), inflammation markers (CD192, TGFB) and stem cell markers (SOX2 and CD133).

Results:

Overall, the mean and standard deviation values for tumor cells were 85%±11% in the stained samples for the Panel *1 Canto* and 73%±17% for the Panel *2 Fortessa*, allowing both panels to identify immune populations (12%±9% vs. 20%±16%). The mean percentage of myeloid cells detected was 11%±9% and 19%±15% identifying not only APCs (8%±7% vs 13%±14%), but also neutrophils (4%±7%) and myeloid derived suppressor cells (MDSC) (2%±2%). For the lymphoid origin cells, Panel *1 Canto* detected only CD4 fraction. However, Panel *2 Fortessa* allowed the simultaneous identification of TCD4+ lymphocytes (0.2%±0.7%), CD8+ (0.6% ±0.7%), extremely low numbers of regulatory T-cells, B-

lymphocytes (0.1%±0.2%) and NK cells (0.1%±0.1%). Finally, the Panel *2 Fortessa* allows to characterize the tumor fraction, being the mean of astrocytic cells, 92%, and stem cells, 26%.

Conclusion(s):

This study demonstrates that: i) the simultaneous use of several markers allows to characterize and quantify different cellular fractions in GBM. ii) The Panel *2 Fortessa* was more accurate than Panel *1 Canto* identifying higher number of cellular subpopulations. iii) Tumor, stem and immune cells were distinguished simultaneously in all analyzed GBM, being APCs the main immune population found in these tumors.

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Considering Systemic Mastocytosis in the differential diagnosis of Acute Myeloid Leukaemia: a case report

Ligia Gabriela Gabrie Rodríguez[1], Tamara Mateu-Albero[1], Laura Esparcia-Pinedo[1], Ildefonso Sánchez-Cerrillo[1], Ángela Figueroa-Álvarez[2], Víctor López-Huete[1], Cecilia Muñoz-Callejas[1]
[1]Inmunology Service Hospital Universitario de La Princesa, Madrid, Spain, [2]Haematology Service Hospital Universitario de La Princesa, Madrid, Spain

Introduction:
Systemic mastocytosis (SM) is a heterogeneous clonal disorder, characterized by abnormal mast cell infiltration of bone marrow (BM) and/or extracutaneous organs. A subtype of SM is associated with haematological neoplasms such as myelodysplastic syndrome, acute myeloid leukaemia or non-Hodgkin's lymphoma. The distinctive immunophenotypic characteristic of both normal and pathological mast cells is the strong reactivity of CD117.

Clinical case presentation: We present the case of a 69-year-old woman who suffered from diarrhoea and persistent eosinophilia in the past 8 months. Eventually, the development of anaemia and thrombocytopenia was the reason for referring the patient to the haematology department of our hospital. Upon arrival a peripheral blood smear revealed 43% blasts of myeloid morphology with frequent cup-like nuclear invagination.

Immunophenotypic strategy: An 8-colour flow cytometry (FC) immunophenotyping panel for screening was performed on a BM sample to assign the linage of the blasts and then we used our in house panel to characterize acute myeloid leukaemia.

Definitive diagnosis: We identified two distinct blast cells populations by FC: the first one represented 32% from the whole cellularity and expressed phenotype SSClow CD45dim, CD34bright, HLA-DRbright, CD64+, CD105+, CD123+ and CD33dim, while the rest of the molecules, including those associated with either myeloid, B or T lineages, were negative. The second population constituted around 40% of the BM sample, that was quite homogeneous for FSC/SSC parameters, and expressed SSC intermediate CD45bright, CD34-, CD117+, CD33bright, CD13heterogeneous, CD38dim, CD64+, HLA-DR-/dim, negative for CD15, CD14, CD16 and CD11b, and for molecules specific for both B and T lineages. The phenotype of this second population clearly fitted with that of acute promyelocytic leukaemia were it not for the negative cytMPO. Interestingly, the morphologic examination of the BM aspirate showed a diffuse infiltration with 80% atypical mast cells and 10% myeloid blast cells. We, therefore, included a panel for mast cell characterization and found that the second population was CD203c+, CD69+, CD35+, CD2+ and CD25+, confirming its neoplastic nature. The sequencing study revealed mutation p.Asp816Val of cKIT and on RUNX1 and ASXL1 which are associated with SM and acute leukaemia, respectively. The patient was diagnosed with SM associated with acute leukaemia with mutated RUNX1.

Treatment and evolution: She received different chemotherapy treatments, initially with midostaurin, corticoids and azacitidine; then cladribine due to refractoriness. Eventually the patient was treated with decitabine and gentuzumab since both pathological populations were CD33+, but no response was obtained. The patient died 7 months after being diagnosed. **Discussion:** SM should be always considered in the differential diagnosis of acute myeloid leukaemia when moderate or low expression of CD117 is detected, since cases of poor prognosis SM are associated with a more immature phenotype with decreased reactivity for molecules associated to mast cells. This is especially relevant for those flow cytometry laboratories with no immediate access to the information from other diagnostic tests and/or clinical information.

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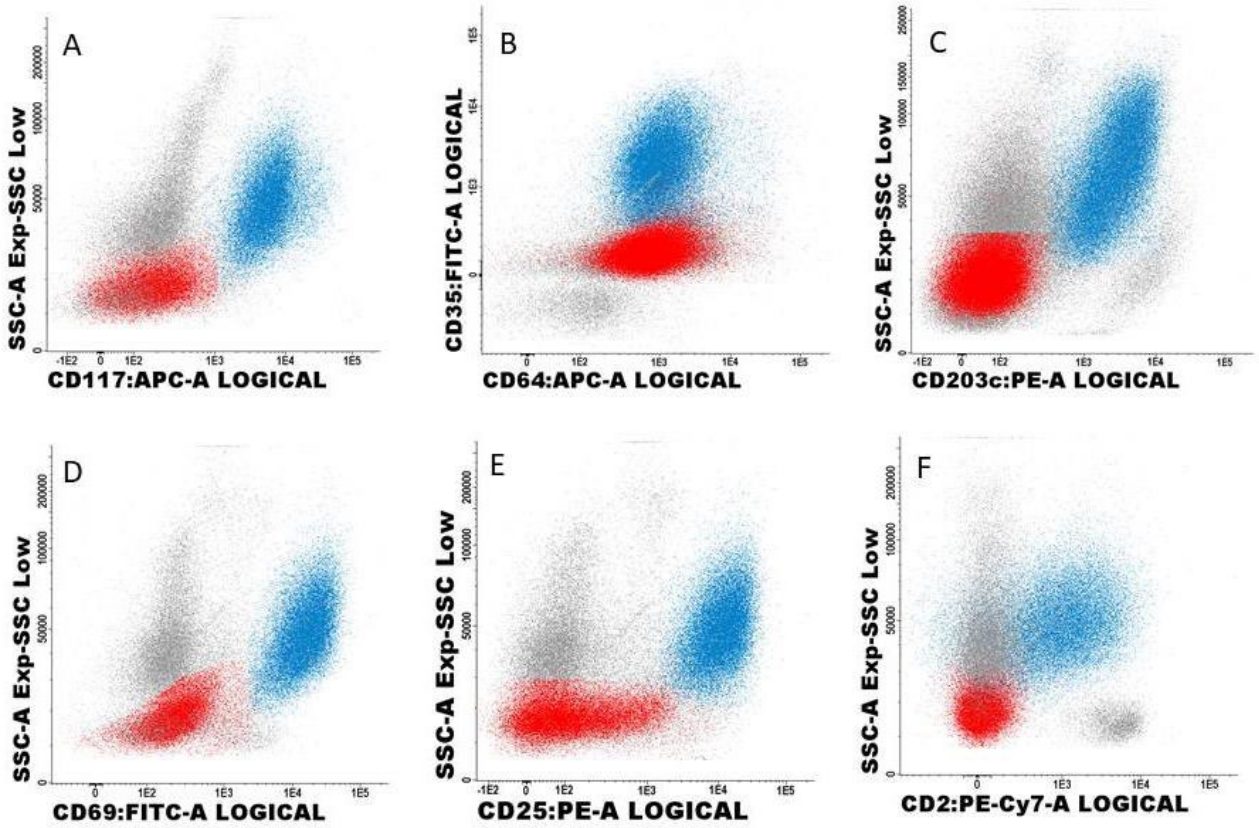

Figure 1. Immunophenotypic analysis of blasts (red) and neoplastic mast cells (blue) showing the expression of CD117 (A), CD35, CD64 (B), CD203 (C), CD69 (D), CD25 (E) and CD2 (F).

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Bronchoalveolar Lavage in the Diagnosis of Pulmonary Diseases: Flow Cytometry Findings. A 2-Year Study

Andreia Monteiro[1,2], Mariana Feijó[2], Sara Correia[2], Maria Jesus Valente[2,3], Patricia Amantegui[1,2]
[1]Serviço Patologia Clínica, Centro Hospitalar Universitário Cova da Beira, Covilhã, Portugal, [2]Faculdade Ciências Saúde - Universidade Beira Interior, Covilhã, Portugal, [3]Serviço Pneumologia, Centro Hospitalar Universitário Cova da Beira, Covilhã, Portugal

Introduction:

The cytological and flow cytometric analysis of bronchoalveolar lavage (BAL) is amply done, based on the clinical presentation and radiological findings. Although, the diagnostic value of BAL is still challenging. The present work aims to evaluate the relationship between BAL flow cytometry's results and the final diagnosis in pulmonary diseases (PD).

Methods:

Between 2017 and 2018, the clinical pathology service of our institution performed 158 BAL. The frequency of total lymphocytes, $CD4^+$ and $CD8^+$ T cells, NK cells, B cells and macrophages were assessed by flow cytometry based in surface antigen expression. The study population includes 88 male and 70 females, with age comprised between 17 and 87 years and mean age of 62 years, 94 non-smokers, 32 ex-smokers and 25 smokers. This population includes cases of pulmonary neoplasm (PN), pneumonia, idiopathic pulmonary fibrosis (IPF), sarcoidosis, bronchial asthma (BA), hypersensitivity pneumonitis (HP) and chronic obstructive pulmonary disease (COPD). All data were treated anonymously, under a confidentiality agreement. Statistically significant differences ($P<0.05$) between the tested groups were obtained by Kruskal-Wallis test followed by Mann-Whitney test.

Results:

The main outcomes of our study were: (a) In sarcoidosis, pneumonia and PN, total lymphocytes were significantly increased; (b) An elevated CD4/CD8 ratio was observed in sarcoidosis, being statistically significant in comparison to IPF, COPD, BA, pneumonia, and PN; (c) A general decreased frequency of macrophages, being the lowest were from sarcoidosis and the highest from IPF.

Conclusion(s):

The elevated correlation between smoking habits and COPD, as well as the high CD4/CD8 ratio in sarcoidosis were corroborated with several studies. Since sarcoidosis is a granulocytic disease, the increased frequency of lymphocytes was expected. In the case of pneumonia, the lymphocytosis can be justified by the fact that all study cases were in acute infection phase. General reduced frequency of macrophages can be attributed to the utilization of percent values and, since the most representative populations in BAL were lymphocytes and macrophages, the increase of one of these cells causes the decrease of another. This observation is enforced by the remarkable inversion of results when comparing total lymphocytes' and macrophages' frequencies of each condition. In the case of PN and PH, the heterogeneity of the groups does not allow to make solid conclusions. Although preliminary, our results highlighted the importance of BAL's cytometric analysis in differential diagnosis of PD.

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Flow cytometry: identifying biomarkers for the diagnosis of organ-specific autoimmune diseases and response to treatment

Aina Teniente-Serra[1], Marco A Fernandez[2], Berta Soldevila[3], Eduarda Pizarro[4], Cristina Ramo-Tello[5], Eva M Martinez-Caceres[1]

[1]Immunology Department, Lcmn, Hospital Universitari Germans Trias I Pujol, Badalona, Spain, [2]Flow Cytometry Facility, Germans Trias i Pujol Research Institute (IGTP), Campus Can Ruti, Badalona, Spain, [3]Endocrinology Department, Hospital Universitari Germans Trias i Pujol, Badalona, Spain, [4]Endocrinology Department, Hospital de Mataró, Mataró, Spain, [5]Multiple Sclerosis Unit, Universitari Germans Trias i Pujol, Badalona, Spain

In the last years, the significant development in the field of biomedical research has lead to the need to define new biomarkers for diagnosis, prognosis or monitoring of diseases. Multiparametric flow cytometry has been positioned as one of the most useful technologies for monitoring immune-mediated diseases.

For this purpose, we designed an exhaustive flow cytometry panel which allows to analyse minor lymphocyte subpopulations in peripheral blood and validated it in several autoimmune diseases.

1) We found changes in peripheral blood lymphocyte compartments of type 1 diabetes patients at onset of the disease.
2) In Graves' disease patients, a different pattern of lymphocyte subpopulations was identified in patients clinically stable who maintain the presence of anti-TSH autoantibodies, compared to those without autoantibodies.
3) In Multiple sclerosis, we found changes in lymphocyte subpopulations identified in untreated relapsing-remitting patients and progressive forms compared with healthy donors. The influence of immunomodulatory therapies on lymphocyte subpopulations was also analysed in a cross-sectional study.

Analysing the influence of therapies on lymphocyte subpopulations, we identified in a prospective study that Multiple sclerosis patients treated with fingolimod had different patterns of subpopulations, able to discriminate responders versus non-responders to the therapy, in a 12-month follow-up.

In conclusion, characterization of minor lymphocyte subpopulations in peripheral blood, by multiparametric flow cytometry, is a useful tool to identify potential biomarkers for the diagnosis and response to treatment of organ-specific autoimmune diseases, and by extension to other immune-mediated diseases.

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Interferon-beta treated-multiple sclerosis patients exhibit a decreased ratio between immature/transitional B cell subsets and plasmablasts

Andreia Monteiro[1,2], Pedro Rosado[3], Luiza Rosado[2,3], Ana Mafalda Fonseca[3], Artur Paiva[4,5,6]

[1]Serviço Patologia Clínica, Centro Hospitalar Universitário Cova da Beira, Covilhã, Portugal, [2]Centro de Investigação em Ciências da Saúde, FCS-Universidade da Beira Interior, Covilhã, Portugal, [3]Serviço de Neurologia, Centro Hospitalar Universitário Cova da Beira, Covilhã, Portugal, [4]Unidade de Gestão Operacional de Citometria, Serviço de Patologia Clínica, CHUC, Coimbra, Portugal, [5]Coimbra Institute for Clinical and Biomedical Research (iCBR), Faculdade de Medicina Universidade de Coimbra, Coimbra, Portugal, [6]Escola Superior de Tecnologia da Saúde de Coimbra, Coimbra, Portugal

Introduction:

Multiple sclerosis (MS) is an inflammatory disease of the central nervous system. MS has long been considered a 'classical' T-cell-mediated autoimmune disorder. However, the involvement of the humoral immunity has always been present by intrathecal synthesis of oligoclonal bands (OCB) in the cerebrospinal fluid, but not in serum. OCB are the most consistent immunodiagnostic feature and hallmark immunologic finding in MS.

Recently, the introduction of B-cell-depleting therapies, which rapidly reduce B cells and eliminate their pathogenicity in MS, demonstrates a strong efficacy in relapsing-remitting (RR)MS, despite their inability to deplete CD20 negative circulating plasma cells.

There are still many unresolved questions surrounding MS and the B cells in this disease. The aim of this work was to quantify circulating B cell subsets in remission and relapse RRMS patients treated with IFN-□ and compare to healthy controls.

Methods:

38 patients with RRMS, 30 of them in remission and 8 in relapse were included in this study, as well as, 20 healthy age- and gender-matched volunteers. By flow cytometry, based on the surface expression of CD19, CD20, CD27, CD38, CD45 and intracytoplasmic staining of IgG, IgA and IgM were identified and characterized the following B subsets: immature/transitional, naïve, $CD27^-$ and $CD27^+$ memory cells and plasmablasts. The results were analyzed using Infinicyt software. Mann-Whitney U test (average ± standard deviation; median) was used for statistical evaluation.

Results:

The most relevant findings were a significant increase of plasmablasts and a decrease of immature/transitional B cells, resulting in a decreased ratio between those cells in relapse RRMS patients, together with an increase of $CD27^-$ and $CD27^+IgM^+$ memory B cell subsets in both phases of the disease.

Conclusion:

These alterations point to an active B cell response, particularly in relapse, and the immature/transitional B cells ratio could constitute a good biomarker of relapse in patients that underwent IFN-beta treatment.

It was seen that participation of B cells in RRMS goes far beyond antibody production alone. Recent data from a bidirectional exchange of B cells through blood-brain-barrier makes the study of B cell subsets even more relevant to be useful for monitoring the disease activity in RRMS patients.

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Immunology reconstitution post-trasplantation: much to discover

María Sánchez[1], Agustín Penedo[2], Yesenia Jiménez[1], Raquel Gonzalo[2], Susana Castañón[2], Selene Ledesma[2], Juan Manuel Alonso[2], Jose Luis López[2], Cristina Serrano[1]

[1]Department of Immunology. Fundacion Jimenez Diaz Hospital, Madrid, Spain, [2]Department of Haematology. Fundacion Jimenez Diaz Hospital, Madrid, Spain

Introduction:

Allogeneic hematopoietic stem cell transplantation (a-HSCT) is used in the treatment of hematological diseases. A critical immunological situation is generated, which entails an increased risk of infections, viral reactivations and even to graft versus host disease (GvHD) that compromising the clinical evolution. In this work we have analyzed lymphocytes subpopulations, focused on NK and T cells subpopulations: $TCD4_{naive}$, $TCD4_{Tcm}$, $TCD4_{Tem}$, $TCD4_{TEMRa}$, $TCD8_{naive}$, $TCD8_{Tcm}$, $TCD8_{Tem}$, $TCD8_{TEMRa}$, $TCD4^{+}TCD8^{+}$, $TCD4^{-}TCD8^{-}$, within the first three months after transplantation, in order to describe possible changes between patients and clinical events.

Methods:

Analysis of lymphocytes subpopulations was done by flow cytometry in patients with a-HSCT on day 0, +15, +30, +100 and +180. An 8-color monoclonal antibody combination was dropped over peripheral blood samples. Relevant data was collected from clinical history.

Results:

15 patients, 67% men and 33% women, 27-69 years and whose diagnoses are distributed in: 4 non-Hodgkin lymphomas (NHL), 6 acute leukemias (AL) (3 myeloid and 3 lymphoid), 4 refractory anemia with excess blasts (RAEB) and 1 aplastic anemia (AA).

At transplantation, 80% of the patients had lymphopenia (≤1000cells/ul), although all populations under study were represented. 46% of patients had less than 200 TCD4/ul. Both, CD4 and CD8 lymphocytes presented an adequate distribution in naive, memory and effector cells.

Only the count of $TCD8_{Tem}$ previous HSCT was significantly higher ($p < 0.05$) in patients with positive CMV (23.5 cells/ul) versus negative CMV group (9 cells/ul).

NK cells on day 0 were significantly higher in NHL versus RAEB: 226 cells/ul (30.8%) and 39 cells/ul (6.6%) respectively. The percentage of $TCD8_{TEMRa}$ was higher in the group consisting of RAEB and AML (23%) compared to NHL (4.35%) ($p<0.05$). No differences were found when they were studied separately.

After a-HSCT, lymphocytes decrease. $TCD8_{naive}$ and $TCD8_{TEMRa}$ cells reach their lowest levels on day 15, while $TCD4_{naive}$ and $TCD4_{TEMRa}$ on day 30. After reaching their minimum levels they increase progressively. On day 100 T_{naive} cells continue increasing but T_{TEMRA} cells decrease again. On the other hand, $CD4^+CD8^+$, central memory T (Tcm), effector memory T (Tem) and NK cells follow a similar pattern to $TCD8_{TEMRa}$. The $TCD4^-CD8^-$ seems more like $TCD8_{naive}$.

Conclusion:

Patients affected by RAEB are the ones with the most immunosenescence data before HSCT.

Immune reconstitution can help us to define personalized prophylactic protocols or even to find biomarkers of viral reinfection that might increase patient safety until the immune reconstitution had been completed.

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Flowcytometric analysis of B Cell subpopulations in patients with Common Variable Immunodeficiency and patients with Immunoglobulin A Deficiency

Rocío Valencia Pereira[1], Georgina Cortés Sierra[2], Aitana Martínez Rodríguez[1], Emilio Doblaré Castellano[1], Oana Irina Sobieschi[1], Josefa Melero Ruiz[1]

[1]Hospital Universitario De Badajoz, Badajoz, España, [2]Universidad de Extremadura, Badajoz, España

Introduction:

Common variable immunodeficiency (CVID) and Immunoglobulin (Ig) A deficiency are primary immunodeficiency disorders. CVID is characterized by low serum levels of IgG, IgA and/or IgM whereas IgA deficiency is characterized by only decreased serum IgA. CVID shares some features with IgA deficiency such a defect in B cell differentiation. Moreover, progression from IgA deficiency to CVID has been reported in some cases.

Memory B cells are decreased in peripheral blood from patients with CVID. To check whether patients with IgA deficiency have any defect in memory B cells, we have studied 10 adult and healthy controls, 13 adult patients diagnosed with CVID and 9 adult patients with IgA deficiency.

Methods:

One hundred microliters of EDTA-anticoagulated whole blood from each subject was incubated for 30 min at room temperature with the following monoclonal antibodies panel: IgDFITC, IgMPE, CD19PC7, CD27APC, CD38APC-H7, CD21HV421, CD45HV500. Data acquisition was performed using a BD FACSCanto II flow cytometry and data analysis was done using Infinicyt analysis software (Cytognos SL).

B cell phenotyping distinguishes different circulating subpopulations: class-switched memory B cells, marginal B cells, transitional B cells, plasmablasts and $CD21^{low}$ B cells.

Statistical comparisons between groups were made using the Student t test.

Results

CVID patients had lower percentages of class-switched memory B cells compared to control subjects (mean 1,96±1,50 vs 15,21±7,23; p<0,0001) as well as lower levels of marginal B cells mean (8,58±7,65 vs 20,46±11,44; p<0,007). In IgA deficiency patients, no significant reduction in percentages of these B cell subpopulations was found (mean 12,17±8,43 and 16,49±7,76 respectively).

Three IgA deficiency patients (33%), had reduced levels of class-switched (3,3%, 2,5% and 4,9%, respectively) and marginal B cells (8,75%, 8,5% and 8,5%). Indeed, two of them showed serum IgG concentrations lower than in rest of IgA deficiency patients. Autoimmune disorders were found in only one of them.

IgA deficiency patients showed differences in levels of plasmabasts and $CD21^{low}$ cells compared with the control group (low statistical significance).

Conclusion:

Analysis of B cell subpopulations is a standard procedure in the diagnosis of CVID. Although it would be necessary to carry out prospective studies and increase the number of patients, these results suggest that analysis of B cell subpopulations could be useful to detect those patients with IgA deficiency with worse clinical phenotype or who could progress to CVID.

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Chemokines analysis and immunophenotyping by flow cytometry in subjects with hepatitis C before and after treatment with Direct-Acting Antivirals

Fernanda Winckler[1], Aline Braz[1,2], Liciana Silveira[3], Vanessa Paixão[1], Marjorie Golim[2], Giovanni Silva[1]
[1]Botucatu Medical School - Unesp, Botucatu, Brazil, [2]Flow cytometry laboratory at Botucatu Clinics Hospital - UNESP, Botucatu, Brazil, [3]Institute of Biosciences - UNESP, Botucatu, Brazil

Introduction:

The treatment of chronic hepatitis C virus (HCV) with Direct-Acting Antivirals (DAAs) promotes change in immune cells response in those that achieve sustained virological response (SVR). **Aim:** To evaluate the basal profile and variation of the immune cells of subjects submitted to treatment with DAAs.

Methods:

Study performed in the Flow Cytometry Laboratory at UNESP - Botucatu Medical School. We included subjects HCV+RNA, naive and treated, genotype 1 and 3, ≥18 years. Samples were collected at the baseline (P1) and 12 weeks after treatment (P2). The following cell surface markers were evaluated: CD45 -PERCP, CD3-FITC, CD16-APC and CD56-PE for NK cells; CD14-FITC and CD16-APC for monocytes. BD Multitest® (CD3, CD8, CD45 and CD4) was used for the analysis of T lymphocytes (T-cells) in tubes BD TruCOUNT. Quantifications of chemokines were performed using the CBA Human Chemokine Kit (CCL2, CCL5, CXCL8, CXCL9, CXCL10) following manufacturer's instructions.

Results:

33 subjects were included for cells analysis and 40 subjects for chemokines. The cell sample analysis presented 18/33 male; the age was 59.18 years ± 9.48. The Analyses of P1 comparing P2 demonstrated a significant difference between TCD4+ (p0.003) and TCD8+ (p. 0,004) cells, which increased after end of therapy and presented a rapid recovery of these cells. Reconstitution of the T-cell compartment and re-differentiation into effector memory cells are results of DAAs therapy, which is probably linked to global consequences for protective immunity, which was different from what happened in the IFN based therapy. There was no significant change observed in the monocyte subpopulation. it was observed that NK^{bright} decreased in P2 (p0.0009) and NK^{dim} increased (p0,004). Previous studies reported that NK cell subset $CD56^{dim}$ and $CD56^{bright}$ become normal rapidly after end of treatment with DAAs therapy taking into consideration the numbers and functionality. Chemokines sample presented 28/40 male and 50% were infected with HCV genotype 1.

Differences were observed in all parameters when compared P1xP2 as follow: CCL2 (p<.0001), CCL5 (p0.0006), CXCL8 (p0.003), CXCL9 (p<.0001), CXCL10 (p<.0001). It was notable that similar levels to those in healthy controls were not reached even after a reduction in the levels of expression in patients with an SVR.

Conclusion:

All parameters performed were improved after therapy with DAAs in SVR patients. However, there was still some inflammatory activity, once that some parameters did not return to normal stage upon clearance of viral infection.

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Dendritic Cell Generation from Peripheral Blood Monocytes

Inês Godinho[1], Patrick Pais[1], Cátia Mendes[1], João Barreto[1], Cátia Rodrigues[1], Carla Azevedo[1], Ana Pires[1], Carlos Palmeira[1], Emília Sousa[1], Gabriela Martins[1]
[1]*Instituto Português de Oncologia do Porto FG, EPE (IPO-Porto), Porto, Portugal*

Introduction:

Dendritic cells (DC) are the most efficient antigen presenting cells of the immune system. They process the antigen and migrate to the lymphoid organs where the antigen is presented. They also control T and B cell activation, the mediators of specific immunity[1]. DC can be generated *in vitro* by inducing the differentiation of peripheral blood monocytes[2,3]. Due to their antigen presenting capacities there has been an increasing interest in its production, namely for *in vitro* T cell activation and polarization studies. The aim of our work was to assay two methods for the generation and the maturation of dendritic cells, in 7 and in 2 days, from the differentiation of monocytes in peripheral blood mononuclear cells (PBMC).

Methods:

PBMC from 10 blood donors were used. The methods applied, for the generation of DC, were the conventional (Classic) 5 day cell culture with GM-CSF and IL-4, followed by 2 days of maturation and the short term (Fast) culture in which cells are cultivated for 24hrs with GM-CSF, IL-4 and IFN-β, followed by 1 day of maturation. In both methods maturation was performed under two different conditions: with TNF-α + IL-1β + PGE2 (TIP)[2] or TNF-α only.

The immunophenotypic characterization on days 0, 2 and 7 was done by multicolour flow cytometry using the following antibodies: CD4, CD45, CD3, CD16+56, CD19, CD8, CD14, HLA-DR, CD86, CD80, CD11c, CD16, CD64, CD123.

Endocytic ability was measured by the FITC dextran uptake at 4and 37 by flow cytometry[4], on day 2 and 7.

Results:

Monocyte differentiation into dendritic cells was equally observed in the two methods. Phenotypic analysis showed reduced expression of CD14 and CD64 and increased expression of DC markers CD123, HLA-DR and CD11c. As expected, an increase in the expression of the maturation markers CD80 and CD86[2,3] was observed and no marked

differences were seen between methods. In relation to maturation conditions, TIP showed better results. The highest cellular complexity was seen, in the Classic cultures, for both maturations. Endocytosis was identical for both methods[4].

Conclusion:

The two methods allowed the generation of mature dendritic cells capable of endocytosis, whose tolerogenic versus immunogenic profile should be evaluated by the cytokines produced. The Fast method can facilitate the production of DC for activation and polarization studies of autologous T cells *in vitro*.

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Alterations of CD4+ T lymphocyte subsets and in their TCR Vb repertoire distribution in early DMARD-naïve rheumatoid arthritis patients

Ana Gómez Lahoz[1], Cristina Bohorquez[2], Ana Pérez[2], Maria Dolores Sosa Reina[1], Lorena Paule[1], Sergio Haro[1], Raquel Oliva[1], María José León[2], Jorge Monserrat Sanz[1], Melchor Alvarez-Mon[1,2]
[1]Dpto. Medicina. Universidad de Alcalá, Alcalá de Henares, Spain, [2]Reumatología. Hospital Principe de Aturias, Alcalá de Henares, Spain

Introduction:

Mechanisms regulating the autoimmune response in rheumatoid arthritis (RA) are not well understood. However, it is known that T CD4$^+$ lymphocytes play a pivotal role initiating and perpetuating the synovial and systemic chronic inflammation found in the disease. However, the potential involvement of these lymphocytes in early RA remains elusive. Methotrexate (MTX) is the most commonly used disease-modifying antirheumatic drugs (DMARD) in RA patients. Unfortunately, less than thirty percent of these patients do not respond to MTX and need to initiate additional treatments. In this study we have examined the numbers of circulating CD4+ T lymphocytes and their naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}) and effector (T_E) subsets, plus their TCR V repertoire distribution, in a population of DMARD-naïve patients with recently diagnosed RA, according to the clinical response to MTX.

Methods:

Numbers of CD4+ subsets (T_N, T_{CM}, T_{EM}, $T_{E)}$ and their TCR V repertoire distribution (24 V families) expression were measured using multiparametric flow cytometry in peripheral blood from 40 DMARD-naïve RA patients and 33 controls. Patients were treated weekly with MTX and the clinical response to the treatment was established after six months of MTX follow up as responders or non-responders.

Results:

At basal pretreatment conditions, MTX non-responder patients CD4+ T_N lymphocytes percentage and counts were significantly decreased with respect to responder RA patients. In addition, MTX non-responder patients CD4+ T_{EM} and T_E lymphocytes percentage and counts subsets were significantly increased with respect to responder RA patients. These differences were studied by a ROC prediction curve, obtaining a 100% of sensitivity for a 75% of specificity. Furthermore, MTX non-responder RA patients Vβ2 T_{EM} CD4$^+$ T lymphocytes percentage were significantly increased with respect to the responder RA patients, meanwhile, these patients showed an expansion in the percentage of Vβ8 T_{EM} and T_E CD4$^+$ T lymphocytes.

Conclusions:

The numbers of $CD4^+$ T_{EM} lymphocytes can differentiate the response of MTX of the DMARD-naïve RA patients. There is a different expansion of T CD4+ effector lymphocytes according to their TCR V repertoire distribution between MTX responder and non-responder RA patients.

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Immune Mechanisms in Acute Coronary Syndrome, its pathological states and associated pathologies

Marta Fernández-Castillo[1], Elena Blázquez, Rocío López-Esteban[1], Irene Martín - Miguel[2], Ana I. Fernández[2], Javier Bermejo[2], Rafael Correa-Rocha[1]

[1]Immune-Regulatory Laboratory of the Institute of Health Research - Gregorio Marañón Hospital, Madrid, Spain, [2]Cardiology Service - Gregorio Marañón Hospital, Madrid, Spain

Introduction

The Acute Coronary Syndrome (ACS) is a cardiovascular disease with a very high incidence, and evidence has been found of the important role that immunity plays regarding ACS. Pathologies such as diabetes or dyslipidemia promote the formation of atherosclerotic plaques, contributing to ACS development. Moreover, plaque formation and tissue damage trigger a reaction from the immune system, recruiting resident and circulating cells to minimize and eventually end the damage. The objective of this study is to elucidate the immune mechanisms underlying both ACS and its associated pathologies, in order to identify immune targets that could reveal some information for the development of new and more efficient therapies.

Methods

Taking advantage of the multi-parameter flow cytometry technology (Gallios, Beckman Coulter), we were able to analyze a huge number of immune populations in 14 patients with ACS using blood samples. Not only the different cell populations were identified, but also the maturation state of these cells, which gives further information of the behavior of the immune system. We checked whether we could find significant immune variations in the cardiac circulation of these patients, if the phenotypes of ACS had a different role in the immune system and, finally, whether diabetes or dyslipidemia played a key immune role in ACS. This study was performed in the Immune-regulation Laboratory of IISGM.

Results

Even though certain differences have been found in frequencies or absolute counts of a few immune subsets, they don't seem relevant regarding cardiac circulation, ACS phenotypes and the diabetic condition of the patients. However, it has been clearly observed that patients suffering from dyslipidemia show significant variations in their immune system. These variations configure a perfectly defined pro-inflammatory phenotype in patients with ACS who also suffer from dyslipidemia, particularly characterized by a notorious increase of T CD4+ effector cells.

Conclusion

These studies suggest that dyslipidemia certainly plays a role in patients with ACS, being associated with an immune deregulation, while the rest of the parameters show no significant relevance. This pro-inflammatory state in dyslipidemic patients with ACS could be essential in their clinical evolution and become a possible marker for prediction and or progression.

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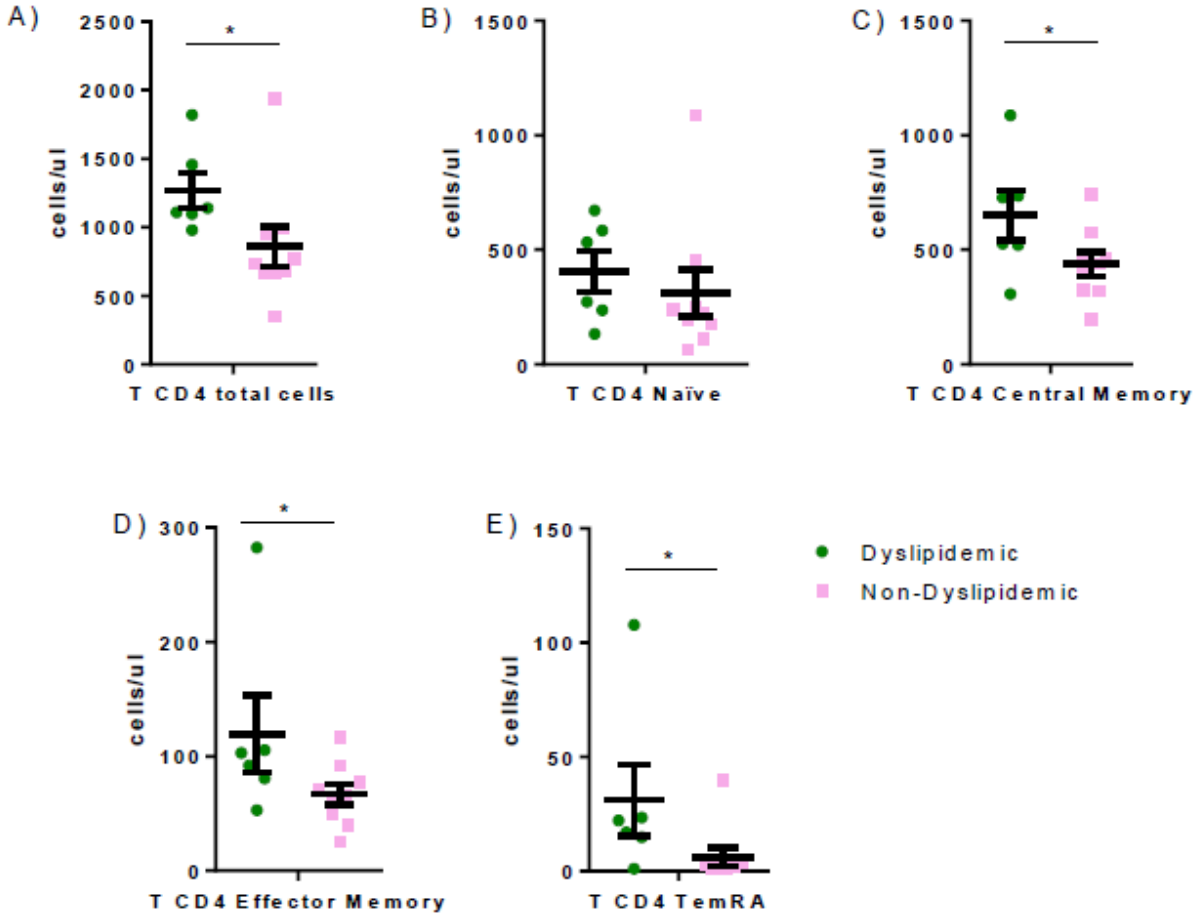

Figure 1. Values of and phenotype T CD4+ cells in dyslipidemic patients with ACS

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Extended immunophenotyping reference values in a healthy pediatric population.

Mónica Martínez-Gallo[1], Marina Garcia-Prat[2], Daniel Álvarez-Sierra[1], Aina Aguiló-Cucurull[1], Sandra Salgado-Perandrés[1], Sara Briongos-Sebastian[1], Clara Franco-Jarava[1], Andrea Martín-Nalda[2], Roger Colobran[1], Isabel Montserrat[3], Manuel Hernandez-Gonzalez[1], Ricardo Pujol-Borrell[1], Pere Soler-Palacín[2]

[1]Immunology Division. Hospital Universitari Vall d'hebron, Barcelona, Spain, [2]Pediatric Infectious Diseases and Immunodeficiencies Unit. Hospital Universitari Vall d'hebron, Barcelona, Spain, [3]Hematology Division. Hospital Universitari Vall d'hebron, Barcelona, Spain

Introduction:

For the accurate diagnosis of immunodeficiencies is crucial to compare patients' immunology laboratory values with age-sex matched controls, yet there is a paucity of normal values for most populations. To define appropriate reference values of extended lymphocyte subpopulations and T-cell receptor excision circle (TRECs) levels in healthy pediatric donors between 1 month and 18 years of age.

Methods:

Extended immunophenotyping values were obtained by analysis of multiparameter flow cytometry panels for the following subpopulations: CD4+ and CD8+ Naive, Effector, Effector Memory and Central Memory, T helper subpopulations and their degrees of activation, T Regulatory cells, Recent Thymic Emigrants (RTE), B Lymphocyte subpopulations (Transitional, Naive, Preswitch-Memory, Switch-Memory, Plasmablasts, CD21low, and Exhausted), and subpopulations for Monocytes, NK cells and Dendritic Cells.

Results:

Median values and the 10th and 90th percentiles were obtained for 32 lymphocyte and monocyte subpopulations, and for TRECs levels in each age group of children. Naive CD4+ and CD8+ T-cell populations tended to decrease with age, with significant difference between the groups, in parallel with the reduction in thymic function assessed by TRECs counts and the recent thymic emigrant population. Relative numbers of Th cell populations tended to increase with age. The percentage of class-switched B cell populations showed a significant increase between the youngest group and the others.

Conclusion:

This study provides essential data for interpreting extended immunophenotyping profiles in the pediatric and young adult populations, which could be of value for the diagnosis of PIDs and immune-mediated diseases, particularly those associated with subtle immunological abnormalities.

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Comparative duodenal intraepithelial lymphogram study between FACSCalibur and FACSCanto II in coeliac disease diagnosis

Carlos Lázaro Perona[1,2], Carlos Lombardía González[2], Judith Vidal Martínez[2]
[1]Hospital Clínico San Carlos, Madrid, Spain, [2]Catlab, Terrassa, Spain

Introduction:

Immunophenotypic identification and quantification of duodenal mucosa intraepithelial lymphocytes (IELs), has been recognised as a valuable resource in order to increase the specificity of the coeliac disease (CD) and refractory CD diagnosis. TCRγδ IELs are invariably increased along time and NK like population is decreased in most CD patients. Phenotypic patterns may be established based on IEL subpopulation percentages.

With the aim of evaluating the interchangeability of duodenal epithelial lymphogram between FACSCanto II digital cytometer and FACSCalibur analogic cytometer (Becton Dickinson), a comparative study between both cytometers was carried out.

Methods:

330 duodenal biopsies from patients with diagnosed or suspected CD were analysed. Biopsies were de-epithelializated to enrich the sample in IELs and then labelled with the following antibodies: CD103 (FITC), TCRγδ (PE), and CD3 (PerCP-Cy5.5), CD45 (APC). The preparations were analysed in parallel by FACSCanto II and FacsCalibur cytometers and the percentage of the following populations were determined over all lymphocytes (CD45+): IELs (CD103+), TCRγδ IELs (CD3+, TCRγδ+), and NK like IELs (CD3-). The interchangeability of results between both cytometers was evaluated by linear regression (Microsoft Excel® 2007) and Bland-Altman analysis (MedCalc®).

Results:

Linear regression slopes between cytometers were as follow: y=0,94x+4,75 (R^2=0.88) for total IELs, y=0,99x+0,16 (R^2=0.99) for TCRγδ T IELs, and y=0,97x+0,88 R^2=0.97 for NK like IELs. In the Bland-Altman analysis, mean ± standard deviation (SD) of the differences between cytometers was 0.46±1.42, 0.09±1.14 and 0.38±2.03, respectively.

Conclusion(s):

A good grade of concordance between cytometers was obtained, especially for TCRγδ T IELs and NK like IELs which are the two parameters with direct implication in CD diagnosis, as total IELs percentage is used for de-epithelialization quality control (studies under 80% of IELs are not informed and de-epithelialization process is repeated).

In the Bland-Altman analysis, small mean-differences were found, with no particular tendency on a specific range of values. However, a relatively wide SD was obtained both for TCRγδ IELs and NK like IELs. These results were partially expected, as flow cytometry epithelial lymphogram is still far from being standardized and professional-dependent analysis might contribute to the difference between equips. While most of stablished clinical patterns were still the same despite the differences, this variability between cytometers might be more relevant for results near cut-off values. For that reason, follow-up and repeat of the biopsy is recommended in those patients with near-limit results for lymphocytic pattern in order to improve the confidence of immunophenotypic evaluation.

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Myeloid-Derived Suppressor Cells in Experimental Autoimmune Encephalomyelitis as predictors of disease severity and tissue damage extent

Virginia Vila Del Sol[2], Rafael Lebrón-Galán[1], María Cristina Ortega[1], Isabel Machín[1], Diego Clemente[1]
[1]Neuroimmuno-Repair Group. Hospital Nacional De Parapléjicos-SESCAM, Toledo, España, [2]Flow Cytometry Unit. Hospital Nacional De Parapléjicos-SESCAM, Toledo, España

Introduction:

Myeloid-Derived Suppressor Cells (MDSCs) are a heterogeneous group of immature myeloid cells with a regulatory role in several immune-related disorders, including Multiple Sclerosis (MS). Previous studies of our group stablished a correlation between the enrichment of splenic MDSCs and the severity of the clinical course, the degree of demyelination and axonal damage, and apoptotic T-cell presence within the CNS of Experimental Autoimmune Encephalomyelitis (EAE) mice. In the present work, we have designed a longitudinal prospective study to analyze the relationship between the percentage of MDSCs in the peripheral blood (PB) and the EAE clinical course.

Methods:

MDSCs, defined as CD11b^{+} Ly6C^{hi} Ly6G^{neg} cells (Ly6C^{hi}-cells), were analyzed in PB from EAE mice at different time-points: before immunization, pre-onset, onset, peak and recovery phases. Aggressiveness indicators, such as Aggressiveness Index (AI), were determined and correlation analysis with the percentage of Ly6C^{hi} in blood was carried out with Spearman test. A second group of animals classified into mild or severe-EAE based on their AI was sacrificed at the peak phase, and demyelination and axonal damage were determined on spinal cords. Moreover, Ly6C^{hi} from mild or severe-EAE mice were sorted and co-cultured with control Tag-iT Violet-labelled T-cells activated with □-CD3 and □-CD28. T-cell proliferation was analyzed 72 h later.

Results:

Our data show that mice with a higher percentage of PB-Ly6C^{hi} cells at the onset develop a milder disease than mice with lower content of Ly6C^{hi} cells, and correlates with several histopathological hallmarks, i.e. myelin preservation and lesser axonal damage. Moreover, the percentage of MDSCs at the peak of EAE inversely correlates with disease severity and show a direct correlation with symptom recovery. Apart from in vivo results, functional experiments show that MDSCs isolated from mild-EAE mice induced a higher inhibition of T-cell proliferation than MDSCs obtained from severe-EAE mice.

Conclusions:

Our data point to MDSC level as a putative biomarker of a less severe clinical course, as well as an indicator of a milder degree of histopathological affectation in the CNS. The fact that MDSCs from milder EAE mice are more effective in the induction of T cell suppression indicate that the relationship between MDSC activity and the severity of the disease course relays not only on the number of regulatory cells, but also in their intrinsic efficacy on T cell control.

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Simultaneous isolation of granulocytes and monocytes-macrophages from sheep and goats from peripheral blood and phenotypic characterization

Noive Arteche-Villasol[1], Valentín Pérez Pérez[1], Julio Benavides Silván[2], Maria del Carmen Ferreras Estrada[1], Marcos Royo González[1], Raquel Vallejo García[1], Daniel Gutiérrez-Expósito[1]
[1]Universidad De León, León, Spain, [2]Instituto de Ganadería de Montaña (CSIC-ULE), León, Spain

Granulocytes and macrophages play a crucial role in the elimination of pathogens during the innate immune response. Its isolation from peripheral blood and *in vitro* culture can be used for the study of the host-pathogen interaction. However, in small ruminants, both cell populations have to be isolated separately and there is no currently method that allows the simultaneous isolation of them. For this reason, in this work an effective method has been developed for the simultaneous isolation of monocytes and granulocytes in sheep and goats establishing its efficiency through the analysis by flow cytometry of its phenotypic characteristics.

Lymphoprep™ was used to separate mononuclear cells (PBMCs) and granulocytes from peripheral blood by collecting two different phases. Subsequently to separate the monocytes from the rest of PBMCs, two isolation techniques were tested: the adhesion method and the immunomagnetic columns. The efficiency of both methods was compared by flow cytometry and after the maturation of monocytes to macrophages, phenotypic characterization was made using CD14, CMH-II and CD11b surface antigens. The morphology was compared in both types of cells. In the case of granulocytes, the purity obtained by this isolation technique was determined according to their morphological characteristics by flow cytometry.

The results showed that the technique of isolation by immunomagnetic columns achieves higher monocyte purity due to expression of CD14, CMH-II and CD11b was higher than 90%, in contrast to the 50% obtained by the adherence. It was also observed that in both techniques the expression of all studied surface antigens in macrophages was greater than 90%. In the case of granulocytes separation with Lymphoprep™ allows their isolation with purity greater than 90% according to their morphology.

In this work an effective protocol which allows the simultaneous isolation of granulocytes and monocytes from peripheral blood with a high purity has been established. Immunomagnetic columns offer better results in the isolation of monocytes. These results set the basis for further studies focused on pathogen-host interaction in granulocytes and macrophages in sheep and goats.

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Influence of gestational age in the distribution of immune cell subsets of preterms

Oihane Perez-Escurza[1], Ana Remesal[2], Elena Blanco[1], Vitor Botafogo[1], Julia Almeida[1], Martin Perez-Andres[1], Alberto Orfao[1]

[1]Department of Medicine, Cancer Research Centre (IBMCC, USAL-CSIC), Cytometry Service (NUCLEUS), University of Salamanca (USAL), Institute of Biomedical Research of Salamanca (IBSAL), Salamanca, Spain and Biomedical Research Networking Centre Consortium of Oncology (CIBERONC) Instituto de salud Carlos III, Madrid, Spain, Salamanca, España, [2]Serv. Pediatrics. University Hospital of Salamanca. Institute of Biomedical Research of Salamanca (IBSAL), Salamanca, Spain, Salamanca, España

Introduction:

Neonatal screening of primary immunodeficiencies and other immune alterations is limited by the scanty information available the immune cells subpopulations in healthy donors at the moment of birth and the lack of reference values.

Methods:

We have analyzed the distribution of >200 immune subsets in peripheral blood of healthy preterms in the first 6 hours after birth, including: 1) maturation (immature/transitional, naïve, memory and plasma cells) and isotypic (IgM, IgD, IgG_{1-4}, IgA_{1-2}) B-cell subsets, 2) CD4+ maturation (naïve, central, transitional and effector memory, and terminally differntiated) and functional (Th1, Th2, Th17, Th22, Th1/17) subsets. EuroFlow SOP high sensitivity methods were used for staining. Data were acquired on a Fortessa X20 flow-cytometer and information analyzed using Infinicyt software.

Results:

We have analyzed samples from 10 preterms from week 32 to 38 of gestation. Although the fetus is an immunologically privileged site, we cconfirm previous observations suggesting that antigen-experienced B- and T-cells can be detected in the neonates already in the moment of birth at extremely low frequencies (0.1-0.01 cells/uL).

Conclusions:

Our data supports that antigen-dependent differentiation take place during fetal development. Accordingly, references value for neonatal immune systems should be defined using donors of different gestational time among other perinatal factors

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Effect of stress on blood fish cell populations determined by FCM quantification

Alberto Alvarez Barrientos[1], Rosa Carrillo del Cacho[1], Isabel Clemente Ramos[1], Bettina Perales Casildo[1], Yolanda Gutiérrez Martín[1], Pilar Torralbo[1], Héctor J. Pula[2]

[1]Servicio de Técnicas aplicadas a la Biociencia, Universidad De Extremadura, Badajoz, Spain, [2]Piscifactoría Vegas del Guadiana, Villafranco del Guadiana, Spain

Introduction:

Stress can modify fish immunological status by affecting blood cells populations. Fish blood cell populations detected by FCM are thrombocytes lymphocytes, monocytes, neutrophils and basophiles. Using flow cytometers with pumps or syringes for taking the sample, direct absolute cell counts can be made. Toxicity can modify fish immunological status by affecting blood cells populations (Maitra et al 2014). The aim of the study is to analyze if stress caused by manipulation and overpopulation can affect blood cell populations from tenches and trout.

Methods:

Blood from caudate vein was collected in heparinized tubes. 50 µL were taken for FCM analysis. Samples were stained with TMRM 2nM in PBS with 0.5%BSA, for 30' at 21ºC. 200µL of PBS 0.5%BSA was added and samples were analyzed in a MACSQuant VYB (Miltenyi Biotech) flow cytometer. Using TMRM fluorescence as discriminator erythrocytes can be avoided and only leucocytes are included in the analysis. Three groups are included, control (no manipulation and fish density of 10 animals per 10 m^3), manipulated (3 manipulations per day) and overpopulation (50 animals per 10 m^3).

Results:

Blood cell populations can be defined by FCM using TMRM as discriminator parameter. By changing the threshold value, erythrocytes can be eliminated of the analysis and blood cell populations can be perfectly defined. Regions are used to delimited thrombocytes, lymphocytes, monocytes and neutrophils-basophiles. Absolute cell quantification, which can be achieved by MACSQuant VYB, gives directly data from each sample, and can be exported to excel, using Flowlogic software (Inivai Technologies). Data obtained demonstrated that manipulation and overcrowding produce decrease in lymphocyte and neutrophil number.

Conclusions:

FCM allows the rapid determination of stress effects on immunological populations from fish blood. This application can be used in fish farms to detect stress situations on fish cultures.

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Detection of Stress in Fish Blood Cell populations by measuring Mitochondrial Membrane Potential and Viability by FCM

Alberto Alvarez Barrientos[1], Isabel Clemente Ramos[1], Yolanda Gutiérrez Martín[1], Bettina Perales Casildo[1], Rosa Carrillo del Cacho[1], Pilar Torralbo[1], Héctor J. Pula[2]

[1]Servicio de Técnicas Aplicadas a la Biociencia, Universidad De Extremadura, Badajoz, Spain, [2]Piscifactoría Vegas del Guadiana, Villafranco del Guadiana, Spain

Introduction:

Stress can modify fish immunological status by affecting blood cells populations. Fish blood cell populations detected by FCM are thrombocytes lymphocytes, monocytes, neutrophils and basophiles. Using flow cytometers with pumps or syringes for taking the sample, direct absolute cell counts can be made. Toxicity can modify fish immunological status by affecting blood cells populations' activity and functionality (Maitra et al 2014). The aim of the study is to analyze if stress caused by manipulation and overpopulation can affect blood cell populations activity from tenches and trout.

Methods:

Blood from caudate vein was collected in heparinized tubes. 50 µL were taken for FCM analysis. Samples were stained with TMRM 2nM in PBS with 0.5%BSA and HOECHST 33258 1µgr/mL, for 30' at 21ºC. 200µL of PBS 0.5%BSA was added and samples were analyzed in a MACSQuant VYB (Miltenyi Biotech) flow cytometer. Using TMRM fluorescence as discriminator erythrocytes can be avoided and only leucocytes are included in the analysis. Three groups are analyzed: control (no manipulation and fish density of 10 animals per 10 m^3), manipulated (3 manipulations per day) and overpopulation (50 animals per 10 m^3). Mean Fluorescence Intensity (MFI) from TMRM fluorescence is analyzed as measure of activity and HOECHST 33258 is used as viability dye to detect death cells and quantify them.

Results:

Blood cell populations can be defined by FCM using TMRM as discriminator parameter. By changing the threshold value, erythrocytes can be eliminated of the analysis and blood cell populations can be perfectly defined. Regions are used to delimited thrombocytes, lymphocytes, monocytes and neutrophils-basophiles. MFI from TMRM and percentage of death cells are acquired from each region using MACSQuant VYB. Data are exported to excel, using Flowlogic software (Inivai Technologies). Results demonstrated that manipulation and overcrowding produce decrease in mitochondrial membrane potential from fish blood cells and increasing death cell percentage.

Conclusions:

FCM allows the rapid analysis of parameters related to stress on immunological populations from fish blood. And detect fast and precisely the alterations on functional parameters on immunological populations from fish blood. This application can be used in fish farms to detect stress situations on fish cultures.

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Flow cytometric characterization of phagocytosis and oxidative burst in whole-blood of dolphins using modified commercial assays designed for human diagnostics

Mar Felipo[1], Alicia Martínez[2], Consuelo Rubio[3], Teresa Álvaro[3], Mónica Valls[3], Carlos Rojo[3], José Enrique O'Connor[1]

[1]Laboratory of Cytomics, Department of Biochemistry and Molecular Biology, University of Valencia, Valencia, Spain, [2]Cytomics Technological Service, Príncipe Felipe Research Center , Valencia, Spain, [3]Comunidad Valenciana Oceanogràfic Foundation, Valencia, Spain

Introduction:

Exposure to toxins, as well as different sources of stress are associated to functional alterations of phagocytic cells. As long-lived cetaceans at the top of aquatic food chain, dolphins may accumulate environmental contaminants. In order to detect immunological alterations in Cetaceans we were interested in adapting to marine species some assays of phagocytic activity in human blood. However, the lack of dolphin-specific antibodies limits the applicability of these assays. For this reason, we have tested on dolphins several available antibodies recognizing human leukocytes. After identifying a suitable marker for dolphin leukocytes, we have adapted three commercial kits for assesing phagocytosis in humans to evaluate phagocytosis and oxidative stress in healthy dolphins living in controlled aquarium environment.

Methods:

Heparinized whole blood samples from healthy common bottlenose dolphins (Tursiops truncatus) were obtained from the Oceanogràfic aquarium (Valencia, Spain). Samples were stained with several anti-human leukocyte-specific CD markers. For functional analysis of phagocytic cells, we used three human commercial systems: FagoflowEx Kit and IngoFlowEx Kit (both from EXBIO) and pHrodo™ Red *E.coli* BioParticles™ Conjugate for Phagocytosis (Thermofisher Scientific). *FagoFlowEx Kit is* designed for examining the oxidative burst with the peroxidase sensor Dihydrorhodamine 123 (DHR123) after stimulation of phagocytic activity with unlabeled *E. coli.* IngoFlowEx is based on measuring the intensity of phagocyte fluorescence after engulfing FITC-labeled *E. coli.* The pHrodo™ Red BioParticles Kit measures phagocytic activity based on acidification of labelled *E. coli* as they are ingested, increasing their fluorescence when the surrounding pH becomes more acidic.

Results:

Human CD11a-PECy5 (Clone HI111, Becton Dickinson) was found to label specifically dolphin leukocytes. Based on this gating, performing the three phagocytosis assays was satisfactory in whole-blood samples without lysis. *We modified* IngoFlowEx *assay by including* the superoxide-sensitive probe *hydroethidine (HE) for the examination of oxidative burst. Also, w*e improved the pHrodo™ Red BioParticles assay by adding DHR123 for the simultaneous examination of oxidative burst.

Conclusions:

These modifications of human kits for the evaluation of phagocytosis also worked successfully on blood of dolphins, thus allowing the evaluation of the ingestion of *E. coli* bioparticles and the respiratory burst in this species. These protocols may be used as functional biomarkers in toxicological and physiopathological studies in marine mammals. This project has been sponsored in part by a pre-doctoral fellowship from the Conselleria d'Educació, Investigació, Cultura I Esport (Generalitat Valenciana) to M F-B.

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A whole blood flow cytometric kinetic assay of platelet activation in healthy dogs (Canis lupus familiaris)

Mar Felipo[1], Alicia Martínez[2], Katiuska Satue[3], José Enrique O'Connor[1]
[1]Laboratory of Cytomics, Department of Biochemistry and Molecular Biology, University of Valencia, Valencia, Spain, [2]Cytomics Technological Service, Príncipe Felipe Research Center, Valencia, Spain, [3]Veterinary Faculty, CEU-Cardenal Herrera University, Alfara del Patriarca, Valencia, Spain

Introduction:

Platelet activation plays a major role in many physiological aspects of hemostasis. Alterations in this process have been associated with pathological conditions leading to either thromboembolic or hemorrhagic disorders. For this reason, the study of platelet activation is important to assess in vivo platelet function and its alterations in veterinary medicine. Furthermore, it is useful to provide predictive risk factors in hemostasis disorders and their therapy. A suitable process for assesing platelet activation is the rise in cytosolic free Ca^{2+} that precedes several activation responses after platelet stimulation, as shape change, aggregation, secretion, and expression of procoagulant activity. These aspects have been extensively studied in human medicine, but not in the veterinary field. Thus, the aim of this study is to apply flow cytometric dynamic assays for platelet activation validated in humans, to small domestic animals, such as dogs or cats.

Methods:

Citrated whole blood samples from healthy dogs were obtained from the Veterinary Clinical Hospital of Cardenal Herrera-CEU University (Valencia, Spain). Samples were tried systematically with several anti-human CD41-PE clones to identify specifically platelets. The Ca^{2+} probe Fluo4-AM (TermoFisher) was used in order to follow platelet activation responses to ADP by a kinetic assay, as previously described by us (Curr Protoc Cytom. 2003 May; Chapter 9: Unit 9.20. doi: 10.1002/0471142956.cy0920s24.) The assay was performed in a CytoFLEX flow cytometer (Beckman-Coulter, CA, USA) using violet side scatter as trigger signal to improve the detection of small particles. Data were analyzed off-line using CytExpert software (Beckman-Coulter).

Results:

During the setup of the functional assay, the clone MEM-06 (Sysmex), reacting against human platelets, was shown to identify specifically canine platelets. Real-time cytometric analysis of Ca^{2+} mobilization demonstrated that ADP triggers platelet activation in dogs, in a similar way to human platelets under similar experimental conditions.

Conclusions:

A commercially available anti-human CD41-PE (Clone MEM-06) can be used to identify platelets in dogs. This allows to perform easily whole blood kinetic assays of platelet activation based on ADP signalling that may be used as functional biomarkers in physiopathological studies and for therapy monitoring in dogs. This project has been sponsored in part by a pre-doctoral fellowship from the Conselleria d'Educació, Investigació, Cultura I Esport (Generalitat Valenciana) to M F-B.

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Flow karyotyping for chromosome sequencing

Eva Julià[1,2], Àlex Bote[2], Erika Ramírez[2], Òscar Fornas[2,3]
[1]Institut Hospital del Mar d'Investigacions Mèdiques (IMIM), Barcelona, Spain, [2]Centre for Genomic Regulation (CRG), The Barcelona Institute for Science and Technology (BIST), Barcelona, Spain, [3]Universitat Pompeu Fabra (UPF), Barcelona, Spain

Introduction:

Flow karyotyping was developed during 70´s to analyze human chromosomes. The strategy was based on their relative DNA content and base pair composition using a double DNA staining with Hoechst-33258 and chromomycin-A3 simultaneously. Chromosome sorting has been used to study variations between human chromosomes, to detect chromosomal abnormalities, to map genes and to generate chromosome-specific libraries. This approach can also be used to detect structural DNA variants such as large genomic deletions, translocations and copy-number variation on specific chromosomes.

The objective of the current work was to use Flow Karyotyping to isolate chromosomes for its subsequent sequencing by Nanopore sequencing technology.

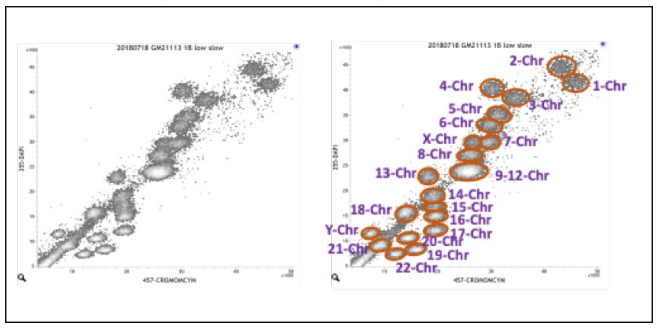
Methods:

Chromosomes suspension were prepared from lymphoblastoid cell line (Coriell, HG02982). Cells were blocked in mitosis by using standard Colcemid procedure. To swell cells and stabilize their chromosomes hypotonic and polyamine isolation buffer were respectively used. Cells were vortexed to liberate the chromosomes for sorting. Chromosomes suspension were stained with chromomycin-A3 and Hoechst 33258 in presence of divalent cations.

Chromosome sorting was performed on BD Influx cell sorter (Becton Dickinson, San Jose, CA) using blue, deep-blue and UV lasers. The setup and instrument performance were optimized using beads by standard procedures. All parameters were collected in lineal mode and analyzed with the BD FACS Sortware.

Results:

We develop a high-resolution bivariate flow karyotyping to isolate human Y-chromosome as shown in the following figure.



The developed flow karyotyping has shown to be highly efficient obtaining DNA to be directly sequenced without amplification by Nanopore sequencing technology.

Conclusion:

We efficiently used bivariate flow karyotyping as an accurate tool to isolate human chromosomes for its subsequent DNA sequencing without amplification on a MinION nanopore sequencing device.

Acknowledgements:

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Hypolipidemic activity of phenolic-rich extract from Pomegranate (Punica granatum L.) in Triton WR 1339 induced hyperlipidemic rats

Lamiae Benchagra[1], Mhamed Hamchoun[1], Abdelouahed Hajjaji[1], Hicham Berrougui[1]
[1]*Department of Biology, Polydisciplinary Faculty, University of Sultan Moulay Slimane, Beni Mellal, Morocco, ,*

Punica granatum L. has been claimed to provide several health benefits. Few studies reported relation-ship between pomegranate consumption and prevention of oxidative stress, diabetes, inflammation, cancer and cardiovascular diseases development. In this study we investigated the hypolipidemic and anti-lipoprotein-oxidation of phenolic-rich extract from *punica granatum* peels. The antihyperlipidemic activity was studied in rat model injected intraperitoneally with Triton WR-1339. The animals were grouped as follows: normolipidemic control, hyperlipidemic, simvastatin and phenolic extract-treated groups. After 24 hours of treatment, serum lipid profiles were investigated using commercially available kits. The administration of the peels phenols rich-extract significantly reduced the serum levels of total cholesterol, triglyceride (TG), and very low density lipoprotein (LDL) as well as the atherogenic index (A.I) and increased the serum high density lipoprotein (HDL) level compared to the Triton WR-1339 induced hyperlipidemic control rat after 24 hours of treatment. The results of the investigation demonstrated that the peel extract of *punica granatum* has potential antihyperlipidemic activity and might be used for the prevention of hyperlipidemia associated disorders.

Keywords:

Antihyperlipidemic activity, *Punica granatum*, cardiovascular diseases, low density lipoprotein (LDL), high density lipoprotein (HDL).

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Validation of Markers to Investigate Breast Cancer Diagnosis by Flow Cytometry

Maria Cláudia Santos Da Silva[1], Sandro Wopereis[1], Laura Otto Walter[1], Daniella Serafin Couto Vieira[1], Amanda Abdalla Biasi Ribeiro[1], Chandra Chiappin Cardoso[1], Iris Mattos dos Santos[1], Braulio Leal Fernandes[1], Renato Salerno Wilkens[1]
[1]*Universidade Federal De Santa Catarina, Florianópolis, Brazil*

Introduction:

Breast cancer (BC) is the second most common cancer worldwide and it has an important impact over healthcare systems. Nowadays, laboratory diagnosis of BC is done mainly by morphological analysis and by immunohistochemistry of the removed tissue. However, these methodologies have some limitations, as they are slow, allow the evaluation of few markers simultaneously and the assessment of a limited number of cells per sample. Therefore, there is a demand for the development of new analytical methods able to support those currently used. So, the aim of this study was to validate flow cytometry immunophenotyping to investigate diagnostic and prognostic markers of BC.

Methods:

Tumor samples from surgical specimens of 54 patients previously diagnosed with BC were first sliced into smaller pieces and then macerated with PBS. Then, the samples were filtered and the cell suspensions obtained were marked with 2µl of 7AAD and antibodies against estradiol receptors α (ERα); progesterone receptors (PR); HER2 and CD45 conjugated with AlexaFluor® 488, APC; PE and V500 in 3; 7; 15 and 1 µl, respectively. After that, samples were acquired using a FACS Canto II (Beckton-Dickinson®) flow cytometer and analyzed with the software Infinicyt 1.7 (Cytognos). In order to determine the expression patterns, the mean fluorescence intensity (MFI) of marked BC cells were compared with the MFI of lymphocytes, which do not express the receptors of interest. For HER2 receptor analysis, a cutoff was done to discriminate normal and superexpression patterns. All the results were compared in terms of sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) with the immunohistochemistry results; and, when necessary, with fluorescence *in situ* hybridization (FISH). This study was approved by the Hospital's ethics committee.

Results:

Results obtained by comparing flow cytometry with immunohistochemistry or FISH were: ERα detection (sensitivity: 75%, specificity: 90%, PPV: 96,7% NPV: 47,4%); PR detection (sensitivity: 72%; specificity: 70%; PPV: 79,3%; NPV: 60,8%); HER2 detection (sensitivity: 80,0%; specificity: 90,2%; PPV: 66,7%; NPV: 94,9%).

Conclusions:

The results obtained so far show the capacity of flow cytometry to differentiate BC markers with agility and safety. However, further studies are necessary to improve the sensitivity of this methodology. We believe that the combination of flow cytometry with morphological analysis and immunohistochemistry, might overcome the individual limitations of each methodology and, therefore, provide more reliable results on a faster and efficient manner. This would result in significant improvements on the diagnosis and prognosis of BC.

Financial support and grants:

This study was supported by the Foundation for Research and Innovation of the State of Santa Catarina (FAPESC/Brazil) and by the Brazilian National Council for Scientific and Technological Development (CNPq/Brazil). Santos-Silva MC is the recipient of a research fellowship from CNPq/Brazil; Ribeiro AAB from scientific research PIBIC/CNPq and W LO from Coordination for the Improvement of Higher Education Personnel (CAPES/Brazil)

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Accreditation according to ISO 15189 standard for bone marrow MM-MRD monitoring by NGF.

Juana Ciudad Pizarro[1], Susana Barrena Delfa[1], Juan Flores-Montero[1], María José Haro Marcos[2], Antonio López Fernández[1], Miriam Fierro de la Fuente[1], Miriam Santos Sánchez[1], Virginia Herráez[1], Alberto Orfao[1]

[1]Servicio General de Citometría- Nucleus. Centro de Investigación del Cáncer (IBMCC-CSIC/USAL-IBSAL). Departamenteo de Medicina, Universidad de Salamanca, Salamanca, España, [2]Servicio de Calidad- Nucleus. Universidad de Salamanca, Salamanca, España

Introduction:

Accreditation (AC) process of a clinical test should be regarded as a useful pathway to achieve and demonstrate the high-quality levels required in clinical laboratories analysis with impact on clinical decision making. At present, few flow cytometry (FCM) tests are accredited, partly due to the absence of validated criteria, controls and standards; and to the continuous advances that condition an increasing complexity. The Euroflow Consortion standarization and automation strategies of validated utility facilitates and support AC of FCM test. Here we document the steps followed at the Cytometry Service (CS-NUCLEUS) of the University of Salamanca to obtain AC according to ISO 15189 of the high sensitive detection of minimal residual disease (MRD) in bone marrow (BM) of Multiple myeloma (MM) patients after treatment using Next Generation Flow (NGF).

Methods and samples:

Implementation of the EuroFlow method (antibodies conjugates and combinations, procedures, instrument's set-up and data analysis) published and validated[1,2], partially simplified AC to a verification demonstrating same level of performance as the reference method. This validation/verification was based on the experimental design under controlled conditions of reproducible assays to measure accuracy and reproducibility of the methodology in both qualitative- (MRD-/MRD+): minimum limit of detection (LOD) of 0.0002% clonal plasma cells (CPC) and; quantitative- (%MRD): lower limit of quantification (LLOQ) of 0.0005% CPC. To this aim we used: one BM from a healthy donor, admixed at three different concentrations (i.e., at LOD; at LLOQ and; at ≈30%) with the MM cell line MM-MM1S as reference material and we assessed the percentage of CPC. In addition, 1 BM sample of MM-patient was used to evaluate reproducibility of the process. Finally, reproducibility of analysis among different analysts was evaluated using 14 MM-patient samples and 8 sample files from the ICS QA exercise. The end-points were: a) LOD of 0.002%, b) sensitivity >99%, confirmation of reproducibility and of veracity (error) within the same order of magnitude (i.e., 1 logarithmic unit).

Results and Conclusion:

The experiments demonstrated a valid performance, i.e., LOD: 0.0002% and LLOQ: 0.0005%. Veracity: 21.34% and CVR (Analysis) of 21.65%. In the comparability between analysts a bias between 15% and 22% and an uncertainty between 0.06% and 0.006% was obtained. Overall, the results allowed granted the accreditation certificate of the NGF BM-MRD assay by the ISO 15189 standard. The adoption of standardized and validated methodologies simplifies obtaining of AC as well as the processes in our laboratories.

1 Kalina T, Flores-Montero J, van der Velden VHJ, Martin-Ayuso M, Böttcher S, Ritgen M *et al.* EuroFlow standardization of flow cytometer instrument settings and immunophenotyping protocols. *Leukemia* 2012; **26**: 1986–2010.

2 Flores-Montero J, Sanoja-Flores L, Paiva B, Puig N, García-Sánchez O, Böttcher S *et al.* Next Generation Flow for highly sensitive and standardized detection of minimal residual disease in multiple myeloma. *Leukemia* 2017; **31**: 2094–2103.

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Work Plan for obtaining Medical Testing Laboratory Accreditation (using ISO 15189)

María Jose Haro Marcos[1], Susana García Saez[1], Juana Ciudad Pizarro[2], Susana Barrena Delfa[2], Juan Flores Montero[2], María Jara Acevedo[2]

[1]Servicio Calidad en Laboratorios. Universidad De Salamanca, Salamanca, España, [2]Servicio de Citometría, Salamanca, España

Introduction:

The decision to embark on the accreditation of a laboratory calls for the need to establish a work plan that will allow this objective to be achieved. The success of the plan depends mainly on the multidisciplinary personnel team involved that, as a whole, provides knowledge and experience with respect to four important features:

5) Organization and characteristics of the laboratory
1) Technical area to be accredited
2) Technical and management requirements of the quality standard
3) Process and requirements specific to the accreditation process

The Department of Laboratory Quality Control and the Cytometry Service of the University of Salamanca formed a collaboration, following an established work plan, that has led to obtaining accreditation (using ISO 15189) in two technical areas (immunophenotyping and molecular biology).

This work plan will be presented including each of the features addressed for achieving the accreditation.

Development:

Once the initial situation was analysed to ascertain the degree to which the requirements were being fulfilled, priorities were established regarding what should be addressed first. The priorities were set based on complexity, i.e., requirements that needed more time to be developed, or those whose impact on other requirements made it necessary for them to be quickly implemented.

Three main lines of work were established: requirements associated with the technical area of immunophenotyping, technical area of molecular biology, management and accreditation of the laboratory in general.

Each line of work was associated with a plan of action that was continuously monitored to ensure that the established target deadlines were met.

Results:

After completing the work plan, external audits were carried out by the National Accreditation Entity. The laboratory, following the usual accreditation procedures, carried out an action plan to correct any of the factors detected that did not comply with the accreditation requirements. These factors were then re-assessed by the National Accreditation Entity and accreditation was obtained.

Conclusions:

The involvement of a multidisciplinary team providing all the necessary skills is essential to successfully complete the process of laboratory accreditation. In addition, a correct and complete initial diagnosis is crucial for defining the work plan and the amount of time need to carry it through.

Finally, it is vital to have a work plan that outlines and prioritizes all of the necessary aspects and details in order to successfully achieve the process of accreditation in the shortest amount of time possible.

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Flow cytometry inmunophenotyping can identify and characterize solid tumour cells in clinical samples: a preliminary study

Covadonga Quirós Caso[1,2], Luis Fernández[1], Héctor Torres[1], Francisco Dominguez[3], Eduardo Iglesias[4], Enrique Colado[1,2]

[1]Hospital Universitario Central De Asturias, Oviedo, Spain, [2]Instituto de Investigación Sanitaria del Principado de Asturias, Oviedo, Spain, [3]Hospital Universitario de Cabueñes, Gijón, Spain, [4]Hospital Universitario San Agustín, Avilés, Spain

Background:

Multidimensional flow cytometry (MFC) has a well-established role in diagnosis and follow-up of hematolymphoid neoplasms (HLN). This contrasts with its widely accepted lack of utility in non-hematolymphoid neoplasms due to low sensitivity and lack of specificity for the final diagnosis.

Methods:

From March 2010 to December 2018, 5182 tissue or biological fluid samples from adult patients were submitted to rule out lymphoma infiltration by MFC analysis in an academic centre. Immunologic studies were performed in a FACSCanto II (Becton Dickinson Biosciences; San Jose, CA). Data analysis was performed with Infinicyt software v2.0 (Cytognos, Santa Marta, Salamanca). Lack of expression of CD45 and definitive myeloid or lymphoid markers was indicative of the presence of non-hematolymphoid cell population and these samples were further characterized. All MFC results were compared to histopathologic final diagnosis. Statistical analysis was performed using SPSS 20.0 software.

Results:

Presence of non-hematolympohid neoplasm was found in 61 samples (1.17%). The origin of samples was: 6 pleural effusions, 40 fine needle aspiration (FNA) and 15 tissue biopsies. Regarding individual markers, MFC analysis confirmed CD56 expression in 1 undifferentiated neoplasm, 1 glioblastoma, 1 Primitive Neuroectodermic Tumor (PNET), 1 lung carcinoid, 1 mesothelioma, 1 neuroblastoma and 47 lung neuroendocrine carcinoma samples, while it was negative in 7 cases (1 breast ductal infiltrating carcinoma, 1 ovarian adenocarcinoma, 1 lung adenocarcinoma, 2 squamous cell lung carcinoma, 1 mesothelioma and 1 prostate adenocarcinoma). Among CD56 positive cases, neuroendocrine carcinomas emerged with a unique immunophenotypic pattern: CD326 (EpCAM), tetraspanin (CD81 and/or CD9) coexpression with variable CD117 expression (positive in 20 samples, dim

positive in 4 and negative in 9 cases of the samples tested for this antigen). CD326 expression was absent in glioblastoma, PNET, neuroblastoma and mesothelioma cases. In samples infiltrated by small round cells, MFC correctly diagnosed final tumor histopathology in 52 of 53 cases.

Conclusions:

These results support the use of flow cytometry as a rapid and valuable technique to evaluate samples from patients with a suspicion of non-hematologic tumors, especially in neuroendocrine carcinomas. MFC immunophenotyping may be an approach for a fast and accurate diagnosis, which should be validated prospectively.

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Meningeal carcinomatosis as a form of presentation of a gastric neoplasm

Beatriz Alvarez Flores[1], Raquel Ramos Corral[1], Nazaret Del Amo del Arco[1], María Sopeña Corvinos[1], Raquel Guillén Santos[1], Fernando Cava Valenciano[1]
[1]*Laboratorio Central de la Comunidad de Madrid. BRSalud, San Sebastián De Los Reyes, Spain*

Introduction:

Neoplastic meningitis consists of the infiltration of the meninges by tumor cells through cerebrospinal fluid (CSF). Meningeal carcinomatosis (MC) is a common complication of systemic cancers, appearing in 3-15% of solid tumors. The presentation of an unknown neoplasm is very rarely.

Clinical case:
A 54-year-old male went to the hospital due to feeling of dizziness, instability and headache during a week that did not improve with vestibular sedatives. He presented awkwardness in movements and slow language. Lacked medical-surgical history of interest.
Methods: CSF sample was used for the differential count; biochemistry and the remaining sample was analyzed by flow cytometry. For the immunophenotyping the next panel was applied:

FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-Cy7	HV450	HV500
KAPPA	LAMBDA	CD19	CD5	CD10	CD20	CD45	CD138
CD2	CD7	CD4	CD8	CD16	CD3	CD45	HLA-DR
CD22	CD23	EpCAM	CD38	CD79b	CD20	CD45	

The cells were adquired using an 8 colors BD FACSCanto II Cytometer and analysed by the Infinicyt Software.

Results:

Blood count and biochemistry within normality. Cytochemical study of CSF: pleocytosis (114 leukocytes / mm3 with polymorphonuclear predominance (70%)), hyperproteinorrachia and hyperglucorrachia. Normal Lactate and ADA. In the differential count, difficult to identify cells (large size, increased nucleus / cytoplasm ratio, loose chromatin, some

multinucleated cells, intense cytoplasmic basophilia and anomalous mitosis) were observed.

The flow cytometric study evidenced 29.84% of non-haematological cells with expression of epithelial adhesion molecule EpCAM and CD138 antigen. These cells showed high Forward and Side Scatter and didn't have expression of surface light chains of Igs. and showed negativity for the rest antigens studied.

Conclusion:

The final diagnosis was gastric adenocarcinoma with leptomeningeal carcinomatosis. The patient was admitted charging of oncology who dismissed systemic and intrathecal treatment. He died fifteen days later.

Discussion:

Positive CSF cytology for malignant cells is the standard method for the diagnosis of MC along with imaging tests such as MRI. Cytology has a high specificity but low sensitivity. This aspect can be improved with flow cytometry. The emergency laboratory played an important role in the orientation of this case since it was the first to observe atypical cells. However, the analysis by flow cytometry confirmed the presence of non-haematological cells and allowed to rule out the presence of a lymphoma on the same day that the lumbar puncture was performed. Flow cytometry is therefore, a highly effective method for the evaluation of carcinoma infiltration of the meninges in CSF.

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Limitations for automated determination of cell concentration directly on Petri culture dishes

Oscar Bastidas[1], José Enrique O'Connor[1]
[1]*Laboratory of Cytomics, Joint Research Unit CIPF-UVEG, Valencia, Spain*

Introduction:

Cell cultures are a common tool in many in vitro biomedical research experiments and diagnostic procedures. Most accepted measurement methodologies require moving the cells from the culture medium to an external counting chamber or measuring device, such as a flow cytometer. In this research we explore the feasibility of performing a cell concentration determination inside Petri dishes, which are one of the most common cell culture containers.

Methods:

Our chosen methodology involved two steps: A first step consisting in experimentally measuring the distribution of mammal cells (cell line 661W) and Flow-Count Fluorospheres (Beckman-Coulter) in suspension on Petri dishes. The goal of this first step is to determine the homogeneity of cell or particle distribution on the dishes. In a second step, we performed an in-silico simulation based on data collected on the first step. We modelled a cell counting process simulating a sampling of the Petri dish using a mathematic model programmed in R. More than one million cell counting simulations were performed using the mathematic model. The main goal of these simulations was to determine how many microscopic fields of the Petri dish needed to be sampled in order to achieve a certain level of accuracy.

Results:

Our data indicate that in order to achieve acceptable cell concentration measurements with an error below 10%, more than 5,000 cells need to be sampled. This means that for a reference concentration of 1 million cells per ml, approximately 25 fields of the Petri dish need to be analyzed using a 10x lens. In order to achieve similar error levels with a Neubauer chamber, where cells are distributed evenly, only 400 cells need to be analyzed.

Conclusions:

Our research indicates that it is possible to determine cell concentration directly from Petri dishes. However, due to the heterogeneous nature of cell distribution, a significant extra effort should be made in order to obtain accurate measurements. This new methodology can be especially useful when the cell culture should not be compromised in order to perform the measurement, or when implemented in a fully automated system, where time and effort required to analyze large amounts of cells are no longer a significant issue.

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Acute myeloid leukemia with mutated NPM1 and acute promyelocytic-like (APL-like) immunophenotype. A case study

Mercedes Rey Rey[1], Carmen González González[2], José Ramón Furundarena Salsamendi[2], Alasne Uranga Aguirregomezcorta[2]

[1] *Laboratory of Immunology-Flow Cytometry. Hospital Donostia (Guipúzcoa, Spain)*

[2] *Hematological Diagnostic Unit. Hematology-Hemotherapy. Hospital Donostia (Guipúzcoa, Spain)*

Introduction:

NPM1 is one of the most common recurrent genetic lesions in acute myeloid leukemia (AML), presenting de novo in adults with normal karyotype. Flow cytometry is key in AML diagnosis and follow-up. The immunological characterization of the blasts is usually quite straightforward, but sometimes blasts from different leukemias display very similar immunophenotypes, being a diagnostic challenge. An example is a subgroup of the AML harboring NPM1 mutation, the so-called "APL-like", in which the phenotype of the blasts mimics the one usually found in acute promyelocytic leukemia (APL). The patient was a 60-year old male with bicytopenia (anemia and neutropenia) and general discomfort with some fever and sweatiness, without bleedings or weight loss.

Methods:

the bone marrow aspirate was stained with the following antibodies: cyCD3-V450/CD45-V500/cyMPO-FITC/cyCD79a-PE/CD34-PerCPCy5.5/CD19-PECy7/CD7-APC/sCD3-APCH7/CD138-V-450/CD71-FITC/CD64-PE/CD117-PECy7/IREM-APC/CD14-APC-H7/HLA-DR-V450/CD16-FITC/CD13-PE/CD11b-APC/CD10-APCH7/CD35-FITC/CD64-PE/CD33-APC/CD71-APCH7/CD36-FITC/CD105-PE/nuTDT-FITC/CD56-PE/CD19-APCH7/CD15-FITC/CD2-PE/NG2-PE. Data were acquired using the Diva Software in a FACSCantoII flow cytometer (Becton-Dickinson) and analyzed with the Infinicyt software (Cytognos).

Results:

16% blasts suggestive of AML were observed upon morphological examination of the blood. The presence of two possible Auer rod bundles along with other morphological findings raised the suspicion of PAL. In the morphological evaluation of the bone marrow aspirate 79.2% blasts were found, most of them with monocytoid aspect, displaying in some cases bilobed nuclei and cytoplasmatic pseudopod formation, with single Auer rods that rarely crossed over. Taken together, the morphological findings were suggestive of AML. Accordingly, 83% myeloid blasts were detected by flow cytometry, with a phenotypic profile closely resembling the one usually found in APL, namely absent expression of CD34 and HLA-DR, displaying CD117 expression, homogeneous bright CD33 and heterogeneous CD13, being negative for CD15. Nevertheless, the clinical symptoms of the patient and the morphological findings of the bone marrow did not fulfil the criteria for APL. Moreover, genetic results were negative for the PML-RARA translocation, being positive instead for the NPM1 and FLT3 gene mutations.

Conclusions:

A fast and proper diagnosis of PAL is crucial, due to the specific treatment (all-trans retinoic acid). Usually, the APL blasts match the immunophenotype described in the literature, making the diagnosis quite straightforward. Nevertheless, in some cases of AML with mutated NPM1 the immunophenotype mimicks the one usually found in APL, thus making the diagnosis challenging and potentially leading to a misdiagnosis (Mason et al.2017, Ferrari et al. 2012, among others). Accordingly it is valuable reporting these cases in order to make an integrated approach to the patients, minimizing the diagnostic pitfalls.