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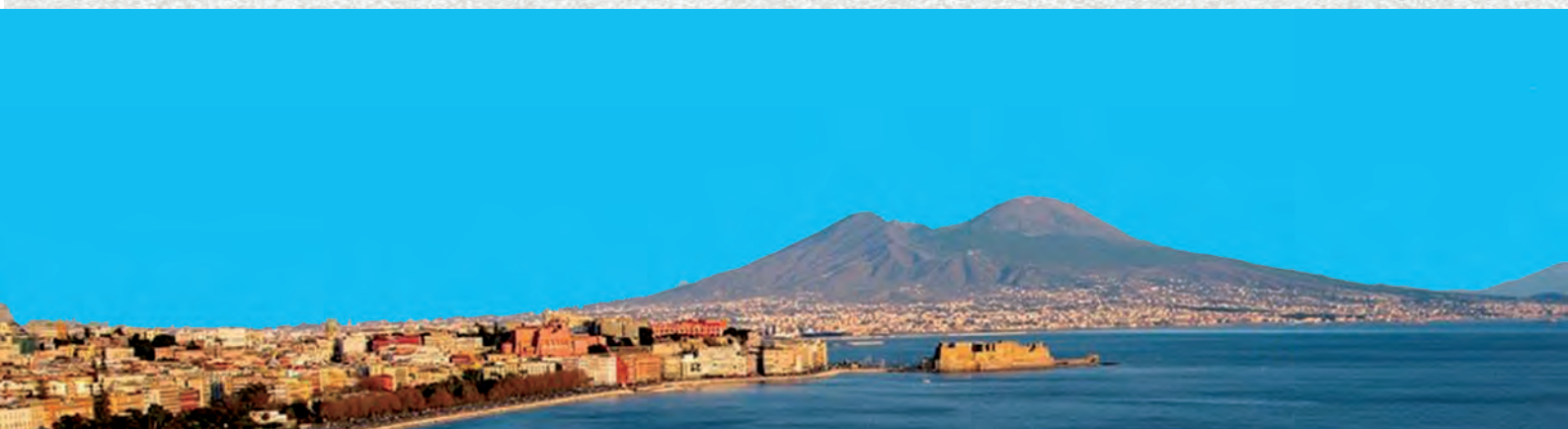
PROCEEDINGS OF THE 41ª NATIONAL CONFERENCE OF THE ITALIAN SOCIETY OF CYTOMETRY GIC



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AGGIORNAMENTI E INNOVAZIONI DELLA CITOMETRIA NELLE APPLICAZIONI CLINICHE E DI RICERCA: ANALISI, GESTIONE DEI DATI E LORO INTERPRETAZIONE

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PROCEEDINGS

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Hematology

A SINGLE FLOW CITOMETRY 8-COLOR TUBE MAY BE USEFUL TO DEFINE THE CELL OF ORIGIN IN FOLLICULAR LYMPHOMA

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Germinal center (GC) is the site of B-cell proliferation, Ig somatic hypermutation, and antigen-driven B-cell selection. Normal GC is polarized into dark (DZ) and light zones (LZ), a distinction that is of key importance to GC B cell selection. Based on the different gene expression profiling (GEP) it is possible to discriminate a DZ or LZ cell of origin (COO) in GC B-cell Non Hodgkin Lymphoma as Follicular Lymphoma (FL) or aggressive B-cell Lymphoma (BCL) as GC-Diffuse Large B cell Lymphoma and high-grade BCL. In particular, in aggressive BCL the definition of a DZ origin with GEP is associated with a worse OS compared to a LZ origin.
Rare data exist about a Flow Cytometric definition of normal GC or GC-COO in aggressive BCL. To our knowledge, no data have been reported about the definition of FL COO using Flow Cytometry (Fc) and the aim of our study was to assess the GC phenotypic pattern in FL.
We analysed 11 biopsies from January 2022 to March 2023 with a histological diagnosis of FL. Cell suspensions were prepared by tissue mechanical disaggregation and they were incubated with ClearLLab B 10C Beckman Coulter (BC) to define the presence of a B-cell mature pathological population. A second 8-color COO tube (IgM-PB/CD45-KO/CD44-FITC/CD10-PE/CD19-ECD/CD27-PC7/CD24-APC/CD38-APCH7) was performed according to Clavarino G et al who distinguished CD44+ CD24+ GC1 LZ from CD44- CD24- GC2 DZ among normal GC B cells from lymph nodes (PLOS One 2016). Data were acquired with DXFlex cytometer (BC).
Data about COO tube revealed a heterogeneous phenotype in FL. In particular CD27, a common GC marker, was expressed in 81.8% (9/11) of CD19+ CD10+ CD38+ FL cells. The analysis of CD44 and CD24 expression on FL cells revealed different phenotypic patterns. In 6/11 patients (pts) (54.5%) we observed a CD44+ CD24+ GC1 LZ origin, in 2/11 pts (18%) we observed a CD44- CD24- GC2 DZ origin. In three pts the GC phenotype was atypical: one pt had a "pre-GC1" CD44++ CD24- phenotype and two pts had a CD24+ expression with a heterogeneous or negative CD44 expression, respectively. Also sIgM expression was heterogeneous. We observed a positive sIgM expression in 66.6% (4/6) of GC1 LZ cases. sIgM was positive in one of two GC2 DZ cases. The two cases with CD24+ and heterogeneous or negative CD44 are both negative for sIgM expression. The only case with a "preGC1" phenotype was positive for sIgM expression.
FL follicles and normal GC share a number of features. FC may help to distinguish different GC phenotypes in FL with a 8-color tube. The analysis of CD24 and CD44 made it possible to identify a different COO as we found a GC1 LZ or GC2 DZ phenotype in most of the pts while in three pts an atypical GC phenotype was detected. To our knowledge this is a first report on the role of FC in the definition of COO in FL. We are currently expanding this study to analyze for associations between the phenotypic COO, the clinical characteristics of pts and the prognostic impact.

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CD79b EXPRESSION IN DIFFUSE LARGE B CELL LYMPHOMA AS ASSESSED BY FLOW CYTOMETRY OF LYMPH NODE BIOPSIES

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The CD79b molecule constitutes the B cell receptor (BCR) complex along with CD79a and surface immunoglobulin. The availability of Polatuzumab, an anti-CD79b antibody drug conjugate, as an effective new target treatment for diffuse large B cell lymphoma (DLBCL) and the scarcity of data about CD79b expression in immunohistochemistry (IHC) and flow cytometry (FC) raised our attention. The aim of our study was to assess CD79b expression in lymph node biopsies with IHC diagnosis of DLBCL using FC.
We analysed 101 biopsies between December 2015 and February 2023. Cell suspensions were prepared by tissue mechanical disaggregation and were incubated with 8 surface markers including the main diagnostic antigens of B-cell lymphomas (KappaV450/CD45V500/CD20FITC/CD79bPE/CD5PerCpCy5.5/CD19PECy7/CD10APC/LambdaAPCH7). Data of the first 45 samples were acquired with BDFACSCanto. Subsequently, 56 samples were acquired with DXFlex (Beckman Coulter). For each antigen we measured Median Fluorescence Intensity (MFI) on pathological CD19+ B cells and CD5+ T cells (negative control population) and we calculated the relative MFI as MFIratio (RMFI) between these two clusters. Analyses were performed separately for the two cytometers.
In the group of 56 patients (pts) analysed with DXFlex the median percentage of CD79b expression on lymphoma cells was 72.5% (2-98). CD79b was strongly positive (>70%) in 50% (28/56), partially positive (range 20%-70%) in 16% (9/56), weakly positive (range 1-19%) in 19.6% (11/56) and less than 1% in 14.2% (8/56) of pts. CD79b expression was observed in residual normal B cells in pts with lymphoma cells negative for CD79b. We observed a positive correlation between CD79bRMFI and clonal light-chainRMFI expression respectively (Spearman R=0.55, p=0.0001). Clonal light-chainRMFI was significantly lower in pts with CD79b expression less than 20% compared to others (Mann-Whitney median value 6.3 versus 30.3 respectively, p=0.01). CD79b expression was not correlated with expression of CD20 and CD10. Similar results were found in the 39 pts analysed with FACSCanto. Spearman test confirmed a positive correlation between CD79bRMFI and clonal light-chainRMFI (R 0.7, p 0.0001) and clonal light-chainRMFI was lower in pts with CD79b expression less than 20% (p=0.006).
We further explored CD79b and light chain expression in two pts without surface expression using intracytoplasmic staining for these antigens. In both pts we observed a positive expression of CyCD79b and a clonal restriction for light chains (Cykappa in one case and Cylambda in one case).
Our results suggest a co-regulation of CD79b surface expression together with other BCR components and indicate a high variability of CD79b expression in DLBCL as previously supposed in literature. It will be interesting whether the quantitative study of CD79b on DLBCL biopsies using FC could be helpful to explore associations between the level of surface expression and response to target therapy.

A THREE-MARKER BASED IMMUNOPHENOTYPE SCORING SYSTEM (ISS) FOR RISK STRATIFICATION OF CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Background. Chronic lymphocytic leukemia (CLL) is a heterogeneous group of clonal B-cell lymphoproliferative diseases with different clinical course, molecular and biological features. Neoplastic B cells frequently have a mature CD5+CD19+CD23+ B lymphocyte phenotype with a weak monoclonal light chain immunoglobulin (SmIg) expression, and low levels of CD20. Risk stratification and prognostication is performed using the CLL-International Prognostic Index (CLL-IPI) or Rai and Binet staging systems. However, more complex integrated prognostic indexes are required to better stratify CLL patients. Here, we designed an immunophenotype scoring system (ISS) using three well-established CLL markers, CD11c, CD38, and CD49d, and its prognostic role in CLL was studied in combination with CLL-IPI and other risk stratification systems.
Materials and Methods. A total of 171 patients were included in this retrospective study after informed consent obtained in accordance with the Declaration of Helsinki. Heparinized or ethylenediaminetetraacetic acid (EDTA) peripheral blood (PB) specimens were directly stained with the following antibodies: CD45; CD4; CD8; CD3; CD56; CD19; CD5; CD23; CD10; CD11c; CD20; CD103; CD38; CD49d; SmIg-Kappa; and SmIg (all from Beckman Coulter, Milan, Italy). Samples were incubated for 20 minutes at 4°C, and then 3 mL of red blood cell lysis buffer was added, samples incubated for 15 min at room temperature, centrifuged, and cell pellets resuspended in 500 μL of PBS. Sample acquisition was performed on a five-color FC500 cell analyzer cytometer (Beckman Coulter) or on a ten-color three-laser Beckman Coulter Navios Flow Cytometer (Beckman Coulter). Post-acquisition analysis was carried out using CPX, Navios tetra software, or Kaluza Analysis Flow Cytometry software v2.1.1 (Beckman Coulter). Instrument daily quality control was carried out using Calibrite Beads or Flow-Check Pro Fluorospheres (Beckman Coulter), and external quality control by UK NEQAS for Leucocyte Immunophenotyping. Samples were run using the same PMT voltages, and at least 1 million events were recorded.
Results. On CLL cells, CD49d, CD38, and CD11c expression was investigated and reported as percentage of positive cells. CD49d+ CLL cells were more frequently CD49d+CD38+ (percent of patients, 48.8% vs 14.6%; $P < 0.0001$), CD49d+CD11c+ (percent of patients, 48.8% vs 27.7%; $P < 0.0211$), or CD49d+CD38+CD11c+ (percent of patients, 34.1% vs 5.4%; $P < 0.0001$). A positive correlation was described between CD49d and CD38 ($r = 0.4190$; $P < 0.0001$) or CD11c ($r = 0.2095$; $P = 0.0095$) expression levels. Next, CD49d, CD38, and CD11c were combined in the immunophenotype scoring system (ISS) for risk stratification of CLL patients. Negativity for CD49d, CD38, or CD11c received a value of 0, while positivity for CD38 or CD11c a value of 1, and positivity for CD49d a value of 3. ISS values ranged from 0 to 5, and each patient received a score. Subsequently, subjects were divided in six groups (from 0 to 5), and clinical outcomes were compared between groups. CLL patients with the highest scores (3 to 5) experienced the shortest TtT compared to those subjects with lower scores. Therefore, we combined this immunophenotype score to CLL-IPI for each patient, and clinical outcomes were compared between groups. Patients with high ISS/high or very-high CLL-IPI displayed the shortest TtT as well as subjects with high ISS and intermediate or low CLL-IPI compared to those subjects with lower scores ($P < 0.0001$). Similarly, our ISS score was combined with the International Prognostic Score for Early-stage CLL (IPS-E), and clinical outcomes were compared. Patients with high ISS and high IPS-E had the shortest 5-year PFS ($P = 0.0336$), and the shortest TtT ($P = 0.0176$).
Conclusions. Our results showed prognostic utility of immunophenotypic markers in combination with clinical, molecular, and biochemistry features in CLL. Moreover, our study added evidence to the need to validate novel complex integrated prognostic systems based on clinical, molecular, and phenotypic aspects.

EVALUATION OF CD4+ AND CD8+ T CELLS COUNT AND RATIO IN CHRONIC LYMPHOCYTIC LEUKEMIA

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Background: Chronic lymphocytic leukemia (CLL) is a mature, antigen-experienced B lymphocyte malignancy. Although significant advances in the understanding and managing of the disease have been achieved, CLL pathophysiology is not yet fully understood, which hampers developing novel therapeutic strategies [1]. The interaction between malignant cells and other immune and non-immune cytotypes within the tumour microenvironment has been recognized as a factor influencing the progression of the disease.
Qualitative and quantitative alterations of T cells in CLL have been reported, and an association between T-cells features and disease progression has been proved [2]. In this context, T cells represent a major immune cellular element modulating the onset and progression of CLL [3]. T cells belong to the adaptive arm of the immune system, displaying accessory and effector functions aimed at defending against pathogens and tumour cells. Once T cells encounter a specific antigen, they undergo activation and, eventually, differentiation into distinct subsets, acquiring either cytotoxic or helper properties. Common changes include, among others, CD8+ T cell expansion and consequent CD4+/CD8+ cell ratio inversion. Understanding the impact of T cell variations in the progression of CLL could improve the management of the disease, but it remains a challenging issue due to some controversies. Namely, it has been reported that an increased number of CD8+ cells is associated with disease progression, shorter time to first treatment and progression-free survival in CLL patients [4]. Oppositely, oligoclonal expansion of CD8+ effector cells has been proved to be associated with disease control in a CLL mouse model, instead of ablation of CD4+ T cells, which did not affect disease progression [5]. Also, it has been suggested that dysfunctional, tumour-specific T cells in CLL patients display immunosuppressive mechanisms, being incapable of eliminating neoplastic cells and promoting the escape of malignant cells from immune surveillance [6].
The expansion of different CD4+ T cell subpopulations in CLL has been reported as well, and their pro-tumoral immunosuppressive activity has been documented [7]. It has been suggested that IFN-γ secretion by Th1 cells provides for a trophic signal for CLL cells, inhibiting apoptosis and supporting survival [7].
Aim: In the current analyses, we assessed T cell count and subsets in 25 CLL Italian patients to evaluate the association of CD4/CD8 ratio and CD4+ and CD8+ absolute count with the expression of CD38 by CLL cells, an established marker of disease severity.
Methods: Twenty-five treatment naïve CLL patients (8 female and 17 male, average age 68 years) underwent T cells flow cytometry evaluation for CD3+, CD4+ and CD8+ T cell percentage by using LST tubes on BD FACSLyric cytometer (BD Biosciences, US). The absolute count was calculated by multiplication with absolute lymphocyte counts obtained on the cell counter. Clinical characteristics (age, sex, B symptoms) were analyzed for any association with T cell features (cell percentages and absolute counts, CD4/CD8 ratio and T cells/clonal B cells ratio). 15 subjects have been enrolled as healthy age-matched controls. Disease severity was defined as the expression of adverse prognosis marker CD38 by clonal B cells [8].
Results: The mean absolute count of CD3+, CD4+ and CD8+ T cells was significantly higher ($p < 0.05$) in CLL patients compared to healthy controls. Based on the CD4/CD8 ratio, patients have been categorized into decreased-, normal- and increased ratio groups, having, respectively, <1.5 (=9 patients), 1.5-2.5 (=10 patients) and >2.5 values (=6 patients). Although an association between T cell features and CD38 expression by B cells has been found, it did not achieve statistical significance.
Conclusions: Alterations in the T cell composition have been extensively described in CLL. However, the exact impact of these changes on disease development and progression remains controversial. Further studies are needed to elucidate whether and how T cell features can be identified as promising targets to develop novel treatment strategies in CLL.
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CONSENSUS FOR FLOW CYTOMETRY CLINICAL REPORT ON MULTIPLE MYELOMA: A MULTICENTER HARMONIZATION PROCESS MERGING LABORATORY EXPERIENCE AND CLINICAL NEEDS

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Flow cytometry is a highly sensitive and specific approach for the discrimination between normal and clonal plasma cells in multiple myeloma. Uniform response criteria after treatment have been established by the International Myeloma Working Group and the EuroFlow Group; less standardized is the way in which these data are reported and no multicenter study has focused on a consensus for the flow cytometry clinical report shared by clinicians and laboratories.

Innovative software tools enable a large-scale data analysis in order to provide accurate and reproducible minimal residual disease (MRD) evaluation, however flow-MRD studies is still strongly linked to the experience of the operator from the pre-analytical phase to the final conclusion of the study. Comparable results can be achieved through a careful harmonization process. Moreover, it is advisable for the clinician to have a precise knowledge of the quality and reliability of the analytical result.

This work, which is based on routine clinical practice, involves 8 expert laboratories and 12 clinical hematology units in the Lazio region. It focuses on the strategies used in real life and, from the analysis of the possible technical problems and pitfalls of flow cytometry characterization in MM, proposes a report designed and shared by flow cytometry operators and approved by clinicians.

From the pre-analytical phase, through sample processing, data acquisition, analysis and evaluation of the potential limitations and pitfalls of the entire process, the study reaches a final conclusion according to the most updated principles and recommendations, providing a clear report that limits subjective interpretations and highlights possible bias in the process better supporting clinical decision-making. The report includes patient personal information; clinical data and biological material; quality control of the sample (cytometric myelogram); flow cytometry analysis of plasma cells and lymphoid populations; the limit of detection (LOD) and quantification (LOQ) for MRD studies; the final comment/conclusion which is a short and clear sentence that states whether the biological sample is suitable for the study and whether a clonal PC population is pre-

sent or absent. All laboratory agreed on the extreme care that must be taken in providing an MRD-negative result, being a positive-MRD highly reliable in MM analysis, if supported by an adequate number of informative events.
A possible limitation in the application of the report proposed may be due to the reporting system available in the laboratory. The development of innovative and certified computer programs dedicated to data reporting and disease monitoring in diagnostic cytometry is ongoing and strongly needed.

PIG-A GENE SEQUENCING STUDY IN A COHORT OF PATIENTS WITH PAROXYSMAL NOCTURNAL HEMOGLOBINURIA SHOWING DIFFERENT SIZE AND DISTRIBUTION OF THE PNH CLONE IN FLOW CYTOMETRY

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Background: Paroxysmal Nocturnal Hemoglobinuria (PNH) is a rare disease and its incidence is around 1–2 per million. PNH is an acquired form of hemolytic anemia, generated by the clonal expansion of a hematopoietic stem cell with a somatic mutation in a gene, called PIG-A and involved in an early step of glycosyl-phosphatidyl-inositol (GPI) anchor synthesis. This mutation causes a total or partial deficiency (resulting in PNH2 and PNH3 clones respectively) of the entire set of GPI-linked proteins in derived blood cells. The current golden standard technique for quantifying PNH clones is multicolor flow cytometry (FCM).
The Italian national FCM archive made by Professor Del Vecchio in 2015 (https://www.clonotecaepn.it) represents the first large-scale unified collection of FCM data and clinical information regarding patients with PNH in Italy. In addition, PIG-A gene has been sequenced to search for the causative mutations of the disease. Thanks to those studies it was possible to denote the presence of unknown and widespread mutations along the entire gene which, in most cases, results in the production of a truncated protein. Currently, to the best of our knowledge, the significance of these mutations in patients in which both the PNH2 and the PNH3 clones are present was not studied.
Methods: The aim of our study was to find possible correlations between specific types of PIG-A gene mutations and different size of the PNH2 and the PNH3 clones. For this reason we investigated, within Clonoteca EPN, patients affected by PNH with PNH2 clones independently distribuited on leukocyte and erythrocyte populations and, subsequently, sequenced the PIG-A gene of these patients by Next Generation Sequencing (NGS). Seven patients affected by PNH (3 male and 4 female) were selected from Clonoteca EPN. Finally, peripheral blood samples were taken from each patient and sent to our laboratory. Analyses were performed by FCM and NGS.
Results: Results obtained from both FCM and NGS analysis, showed a genotype/immunophenotype association. In fact, patients with severe mutations affecting the normal enzymatic activity, showed the presence of a very large PNH3 clone with a small accompanying PNH2 clone (< 20%). Moreover, subjects with no mutations or with mutations that do not affect the activity of the protein showed a large PNH2 clone (> 30%).
Conclusions: The presence of mutations important for PIG-A function in patients with large PNH3 clones on white blood cells allowed us to confirm that the PNH3 clone causes a total defect of the GPI anchor, compared to those with less significant mutations.
Results and Discussion: A panel of 8-12 blood group antigens allows achieving an adequate global sensitivity to detect HBT abuse even many days after its execution. Accurate gating strategy together with high-brightness fluorochromes allow the clear separation of the donor and recipient erythrocyte cells populations. As for ABT, the counting of EMPs is emerging as the most effective strategy to identify the autologous transfusion as it is more sensitive than the monitoring of the decrease in expression of surface antigens in RBC subjected to storage.
Conclusion: Flow cytometry has become a basic technique in doping control for all aspects related to the detection of doping practices with the use of blood cells and at present the only technique suggesting the possibility of developing a direct method of identifying ABT abuse in sport doping.

OPTIMIZATION OF A 10-COLOR STAINING FOR CIRCULATING NEUTROPHIL AND MONOCYTE SUBSETS IMMUNOPHENOTYPING

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Background. Neutrophils, the most abundant circulating leukocytes, are mainly involved in pathogen clearance through three mechanisms: phagocytosis; degranulation; and release of neutrophil extracellular traps. However, neutrophils are not only "pathogen killers" but also they can promote or suppress tumor growth and metastatic processes. These different immunological functions mirror a great heterogenicity of neutrophil maturation and activation status. Two circulating populations can be differentiated and separated by gradient centrifugation: low-density granulocytes (LDGs) and normal-density neutrophils (NDNs). Several observations show the involvement of LDGs in immune dysregulation during autoimmune disorders with an activated phenotype, while NDNs might exert immunosuppressive activities in an arginase-dependent manner. LDGs might also contribute to endothelial cell dysfunction and vascular damage in autoimmune vasculitis, and can be hypo-responsive to anti-myeloperoxidase antibodies despite their high surface expression. Here, a flow cytometric panel that incorporates 11 different markers has been developed and optimized for the efficient identification of the main circulating subsets of neutrophils and monocytes.
Materials and Methods. Whole peripheral blood (PB) specimens were collected in ethylenediaminetetraacetic acid (EDTA) tubes or heparin tubes for immunophenotyping from patients after informed consent obtained in accordance with the Declaration of Helsinki. For immunophenotyping, 50 μL of fresh heparinized whole PB was stained with antibodies according to the manufacturers' instructions. The following antibodies were used: CD56, CD3, CD45, CD34, CD19, HLA-DR, CD15, CD33, CD14, CD11b, and CD16. After 20 min incubation at room temperature, red cell lysis was performed with IO Test Lysing Solution (Beckman Coulter), cells were washed twice with phosphate-buffered saline (PBS) (IsoFlow Sheath Fluid, Beckman Coulter), and then resuspended in 500 μL PBS for acquisition. Samples were acquired on a Navios/EX or a DxFlex cytometer (Beckman Coulter, Brea, CA). Instrument daily quality control was carried out using Flow-Check Pro Fluorospheres (Beckman Coulter), and external quality control by UK NEQAS for Leucocyte Immunophenotyping. Compensation was monthly checked by a Beckman Coulter's Specialist using Flow-Set and compensation kit (Beckman Coulter). An unstained sample was used as negative control, and single-color controls were employed for gain setting. Samples were run using the same PMT voltages, and at least 500,000 events were recorded. Post-acquisition analysis was carried out using Navios EX Software v2.0, or Kaluza Analysis Flow Cytometry Software v2.1.1 (Beckman Coulter).
Results. Cell populations were first identified based on linear parameter (forward scatter area, FSC-A) and CD45 expression, cells were gated, and CD19 and CD56/CD3 expression was investigated. On CD19-CD56-CD3- cells, DR-CD34- population was further identified and studied for CD33 and CD15 expression. On CD33+CD15- neutrophils (normal density granulocytes, NDGs), maturation curve was investigated by CD16 and CD11b expression, and immature CD16-CD11b-, intermediate CD16-CD11b+, and mature CD16+CD11b+ neutrophils were identified. On CD33+ cells, CD14 and CD15 expression was further studied, and CD15+CD14- LDGs were gated. Maturation was investigated using CD16 vs CD11b expression, and CD15 vs CD16. Immature CD16-CD11b-, intermediate CD16-CD11b+, and mature CD16+CD11b+ LDGs were identified, as well as immature CD15+CD16-, intermediate CD15+CD16dim, and mature CD15+CD16+ LDGs were gated. Similarly, monocytes were first gated based on linear parameters, and then classical (CD14+CD16-), intermediate (CD14+CD16+), and non-classical (CD14-CD16+) monocytes were identified. Moreover, on each population, HLA-DR activation status was monitored by measuring median fluorescence intensity of studied marker.
Conclusions. Using this broad phenotyping panel, differences in the main circulating neutrophil and monocyte subpopulations can be easily identified and can be applied for immunophenotyping of patients with autoimmune disorders and hematological malignancies, including myelodysplastic syndromes.

CONCOMITANT DIAGNOSIS OF HAIRY CELL LEUKEMIA AND CHRONIC LYMPHOCYTIC LEUKEMIA: A RARE ASSOCIATION

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A case of concomitant hairy cell leukemia (HCL) and chronic lymphocytic leukemia (CLL) in a 50- year-old man was reported. Two distinct monoclonal B-cell populations were detected by flow cytometry immunophenotypic analyses. The predominant population was consistent with HCL: CD19+high, CD20+high, CD5- , CD23- , CD43- , CD10- , CD103+ , CD25+ , CD11c+ , CD79b+ , CD200+ , FMC7+ , CD22+high, and sIgl+high. A smaller population showed the CD19+intermediate, CD20+low, CD5+intermediate, CD23+, CD43+, CD10-, CD103-, CD25-, CD11c-, CD79b-, CD200+, FMC7+/-, CD22+low, and sIgk+low immunophenotype, consistent with monoclonal B-cell lymphocytosis (MBL), typical B-CLL-like. The patient was first treated with cladribine and then with rituximab and achieved HCL partial remission. Importantly, the high sensitivity of our flow cytometric approach allowed the detection of a small population "P3" in addition to the typical HCL and CLL clones. The P3 clone was absent at diagnosis, appeared after therapy and changed over time, from an HCL-like to a CLL-like immunophenotype. P3 was identified at the last follow up owing to the particular expression (intermediate intensity) of the l light chain in a portion of CD5+ B cells. The P3 population showed an MBL/B-CLL phenotype with a characteristic intensity of CD20 and CD19 markers that was used for the gating strategy. In particular, P3 expressed CD19+intermediate, CD20+intermediate, CD23+, CD5+high, CD25- , CD11c-, CD43+ , CD103- , CD79b+low, FMC7+low, sIgκ- , and sIgl+intermediate immunophenotype. Based on this result, we researched P3 analyzing the previous flow cytometric files. After cladribrine treatment P3 showedan a HCL-like immunophenotype: CD19+ intermediate, CD20+intermediate, CD23-, CD5-, CD25+intermediate, CD11c+, CD103+ , sIgκ- , and sIgl+ intermediate immunophenotype. For this reason, we hypothesized that the P3 population may have originated from the HCL clone. 1 month after the end of rituximab treatment, P3 showed the same HCL-like immunophenotype except for CD20 that was absent. In conclusion, our case is added to the few other cases of synchronous HCL and CLL already reported in the literature and underlines the importance of analyzing chronic lymphoproliferative disorders by highly sensitive diagnostic techniques like the multicolor flow cytometry to evaluate the possible association between HCL and CLL at diagnosis and to monitor minimal residual disease after therapy.

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DYSPLASIA RATE IN MYELODYSPLASTIC SYNDROMES CORRELATES WITH HIGHLY SUPPRESSIVE TREGS EXPRESSING FOXP3-E2 ISOFORM

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Background: An altered immune-tolerance control, mediated by Regulatory T cells (Tregs), has been largely described in MDS. Indeed, in early MDS stage, reduced Tregs levels might favor pro-inflammatory and autoimmune mechanisms, likely able to damage polyclonal hematopoiesis, while in advanced stages, increased Treg-dependent suppression of immune effectors has been observed to foster leukemia progression of the dysplastic clone/s. The expression of the master regulator gene FoxP3 has been largely recognized to be responsible for the immune modulating properties of Tregs. Alternative splicing mechanisms generate two main isoforms of Foxp3, one containing the exon 2 (Foxp3-E2) and another, shorter, lacking the exon 2 (Foxp3-Δ2). The expression of Foxp3-E2 has been recently demonstrated to be essential for Tregs suppressive activity; indeed, Foxp3-Δ2 has been associated with the generation of unstable Tregs and with an autoimmune phenotype in a murine model.
Aims To investigate whether the expression of Foxp3-E2 versus Foxp3-Δ2 isoform by Tregs, resident in the bone marrow (BM), might improve the evaluation of immune tolerance control in MDS. The possibility that such analysis might add a new valuable prognostic biomarker to the evaluation of MDS patients has been also considered.
Methods 27 newly diagnosed MDS patients, according to WHO 2022 and 8 healthy donors were recruited in the study, approved by the Local Ethical committee. BM samples obtained from consenting healthy donors were part of their marrow donation. Patients were categorized according to IPSS-M. Immune profile, Foxp3-E2, overall Foxp3 and ki67 expression by Treg have been evaluated by immune fluorescence and multi-parametric flow cytometry. Mann-Whitney test and Spearman correlation test have been used for statistical analysis.
Results According to IPSS-M, 21 patients were low risk (9 very low, 10 low, 2 moderate low) and 6 were high risk (4 high, 1 moderate high, 1 very high). In high risk MDS patients, Foxp3-E2 expression was significantly increased, as compared to low risk and controls. In addition, in high risk, both Foxp3-E2 and overall Foxp3 CD4+ T cells showed an increased proliferative capability, as assessed by ki67 expression, compared to controls and low risk patients. Foxp3-E2/overall Foxp3 ratio, was significantly decreased in all MDS patients, both low risk and high risk patients, as compared to controls. A significative direct correlation between the BM percentage of CD16-CD11b- dysplastic neutrophils and Foxp3-E2/overall Foxp3 ratio has been identified in the MDS cohort (Figure 1, A). Moreover, the proliferation level of Treg, as evaluated by their ki67 expression, has been observed to directly correlate with the percentage of BM blasts (Figure 1, B). In the end, in low risk patients, Foxp3-E2/overall Foxp3 ratio has been observed to inversely correlate with the activation status of CD8 T cells, as measured trough CD54 expression.
Summary/Conclusions
Here we show for the first time that MDS high risk subjects are characterized by an increased number of Tregs expressing the Foxp3-E2 isoform, thus with a highly suppressive phenotype. More intriguing, the degree of BM dysplasia directly correlate with the prevalence of actively suppressive Tregs and their proliferative status. Understanding how the immune system contributes to MDS physiopathology opens future possibility to identify new prognostic markers and future therapeutic targets, ameliorating MDS clinical management.

HEMATOGONE EXPANSION AFTER ALLOGENEIC STEM CELL TRANSPLANTATION MIGHT BE A SENSIBLE AND EARLY BIOMARKER OF NORMAL HEMOPOIESIS RECOVERY

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Background. Hematogones, B cell precursors with regenerative potential, can repopulate the bone marrow after cell-depleting events, such as high-dose chemotherapy and conditioning regimens for hematopoietic stem cells transplantation (HSCT). In previous studies, hematogones have been proposed as a candidate prognostic biomarker of clinical outcomes after allogeneic HSCT. In this retrospective real-life monocentric study, we investigated the role of hematogones in the prognostic definition of transplanted patients, and their association with other clinical, biological, and molecular biomarkers.

Materials and Methods. A total of 60 consecutive patients who underwent allogeneic HSCT were included in this study. Patients were diagnosed with acute leukemia, myelodysplastic syndromes, multiple myeloma, or non-Hodgkin lymphomas, and received chemotherapy as per international protocols at the Hematology and Transplant Center, University Hospital "San Giovanni di Dio e Ruggi d'Aragona", Salerno, Italy, from 2016 to December 2022. Evaluated parameters were divided in three groups: patients' related factors, including percentage of hematogones after HSCT, age, sex, cytomegalovirus (CMV) positivity, type of disease, comorbidities, cytogenetic abnormalities, clinical and molecular biology; donor's related factors, such as age, sex, and CMV positivity; and transplant procedure-related factors, including source of stem cells, HLA-matching, homogroup, and the number of infused CD34+ cells.

Results. Increased percentage of hematogones at the first re-evaluation post-transplant (median, 104 days) was associated with a higher relapse-free survival (RFS), and those patients showing hematogones >1% of total nucleated cells at the first re-evaluation had a significantly higher overall survival and a lower incidence of Graft versus Host Disease (GvHD) compared to those subjects who did not display hematogone expansion after transplantation. Interestingly, percentage of B cell precursors were significantly higher in female recipients than in males at first re-evaluation and at most recent re-evaluation (median, 542 days) ($p<0.05$ and $p<0.01$, respectively). No associations were described with other factors, such as recipient and donor factors, probability to develop GvHD, or and transplant procedure-related factors. The presence of comorbidities, including hypercholesterolemia/dyslipidemia, diabetes, or gastric diseases, were significantly associated with reduced survival.

Conclusions. Our results confirmed that hematogones evaluated by flow cytometry immunophenotyping could be used as a valid prognostic marker of HSCT outcomes even in a real-life setting. Moreover, appearance of B cell precursors could be a sex-related factor, likely contributing to better clinical outcomes following allogenic HSCT in female recipients.

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HUMAN BONE MARROW 3D NICHE IMMUNOPHENOTYPING

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Background. Hematopoietic stem cell (HSC) maintenance in vitro is challenging because stem cells need cell-to-cell contacts and paracrine signals from stromal microenvironment. 3D-culture systems can mimic physiological tissue architecture thus preserving HSC phenotype. Calcium alginate hydrogels have been successfully employed to mimic bone marrow (BM) niche in vitro and to expand progenitor cells in several 3D bioengineered scaffolds also fabricated by bioplotting technologies. Calcium alginate is a stable and biocompatible biomaterial and can be easily disassembled by calcium chelation resulting in alginate hydrogel dissolution and cell recovery that can be used for further studies, such as flow cytometry immunophenotyping of cultured cells. In this study, we optimized a 10-color staining for immunophenotyping of HSCs, progenitor cells, and mesenchymal stem cells harvested from an ex vivo 3D co-culture system assembled using total BM mononuclear cells (BMMCs) depleted of MSCs co-cultured with BM-derived MSCs in a calcium alginate-based scaffold.
Material and Methods. After scaffold disruption with 50mM EDTA, buffered in 1000mM HEPES, released cells were fixed in 4% PFA for 20 minutes, and then stained with CD90-FITC, CD105- or CD117-PE, CD3- or CD73- or CD33-APC, CD11b-PC7, CD14-APC-A750, CD34-APC700, CD16-PB, CD45-KO, and HLA-DR-FITC (all from Beckman Coulter). Immunophenotyping was performed at day 0, 7, 14 and 21 of culture. Samples were acquired on a DxFlex cytometer (Beckman Coulter), equipped with violet (405nm), blue (488 nm), and red (638 nm) lasers. Instrument daily quality control was carried out using Flow-Check Pro Fluorospheres (Beckman Coulter), and external quality control by UK NEQAS for Leucocyte Immunophenotyping. Compensation was monthly checked by a Beckman Coulter's Specialist using Flow-Set and compensation kit (Beckman Coulter). An unstained sample was used as negative control, and single-color controls were employed for gain setting. Samples were run using the same PMT voltages, and at least 50,000 events were recorded. Post-acquisition analysis was performed with Kaluza software (v2.1; Beckman Coulter).
Results. For 3D culture immunophenotyping, cells were first identified based on side scattered area (SSC-A) and CD45 expression, and myeloid cells were discriminated from mesenchymal stem cells (MSCs) based on HLA-DR and CD73 expression. On CD73- cells, CD33 and CD34 expression was studied, and CD33+ and/or CD34+ precursors were further investigated for CD117 and CD11b expression. On CD33-CD34- cells, CD3+ T lymphocytes and CD14+ monocytes were further identified. Radar plots were employed to visualize dynamics changes of each hemopoietic subpopulation over culture period by displaying CD117, CD16, CD11b, CD33, and HLA-DR markers. Similarly, on CD73+ MSC population, a radar plot was designed using CD73, CD34, CD117, HLA-DR, CD33, and CD11b expression to investigate potential differentiation commitment of mesenchymal cells over culture time.
Conclusions. Using this broad phenotyping panel, both hematopoietic stem and progenitor cells and mesenchymal stem cells can be easily identified and can be applied for immunophenotyping of samples obtained from ex vivo 3D biomimetic co-culture systems.

CONCOMITANT CUTANEOUS COMPOSITE LYMPHOMA AND BICLONAL B-CELL LYMPHOPROLIFERATIVE DISEASE

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Mycosis fungoides (MF) is the main representative of cutaneous peripheral T-cell lymphoma, occasionally associated with myeloma or B-cell chronic lymphoproliferative disorders (BCLPDs). BCLPDs are characterized by light-chain restriction and they are heterogeneous regarding clinical features, morphology, immunophenotype and sites of involvement. Biclonality in BCLPDs is an uncommon event and can be identified by flow cytometry on the basis of different surface light-chain expression, light scatter properties or pattern of surface antigen expression. A 59-year-old man with with a one-year history of plaques and papules on arm and chest's skin and a recent diagnosis of MF was referred to our department for disease staging, The initial blood results showed lymphocyte count of 7.3x109/l and a beta-2 microglobulin of 2.3 mg/l. PB flow cytometry demonstrated an increased percentage of B lymphocytes (49%) with high CD45 expression; analysis of surface immunoglobulin light chain showed a normal kappa/lambda ratio, while a complete immunophenotype of B cells demonstrated expression of CD5, CD20, CD22, CD23, CD43, CD79b, CD200. Notably, two distinct CLL clones were distinct with different expression of CD5 and light-chain restriction; in particular, CD5 dim B-cells (58%) showed kappa-chain restriction while CD5 intermediate B-cells (42%) were lambda-chain restricted. No differences in expression of FMC7, CD23, CD43, CD200 and CD79b were observed. No abnormal expression of CD7, CD5 and CD2 was observed on CD3/CD4 cells. Peripheral and marrow blood morphologic examination showed a heterogeneous lymphocyte population with two prevalent morphological types: one type was characterized by small to medium-sized lymphocytes, with narrow rims of cytoplasm and coarsely clumped nuclear chromatin. The second type was characterized by large lymphocytes with abundant cytoplasm. Biclonal CLL was confirmed on BM aspirate, with a slightly different ratio of two clones. BM biopsy revealed a lymphoid infiltration (75%) of small cells CD20+, CD5+, CD23+/- while CD3, CD4, CD8, Cyclin D1 were negative. Immunohistochemical characterization of skin biopsy documented an epidermotropic lymphoid infiltrate CD3+, CD2+, CD5+, LEF1+, CD4+, CD8-, TIA1-/+, CD30 rare and very weak, IRF4- and a dermal lymphoid component CD20+, CD3-, CD5+, CD23+/-, LEF1+; molecular examination documented a monoclonal rearrangement of both IGH and TCRG. The diagnosis of biclonal CLL and concomitant MF with dermic colocalization of CLL/SLL was made. The mixed morphology could be related to high CD45 expression of B-cells. Our case combines two rare events such as composite cutaneous lymphoma and biclonal BCLPD and it highlights the role of flow cytometry to improve diagnosis of clonality in cases with normal kappa/lambda ratio.

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FLOW CYTOMETRY AS A USEFUL DIAGNOSTIC TOOL IN PNH RARE DISORDER – A CASE REPORT

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Paroxysmal nocturnal haemoglobinuria (PNH) is a rare disorder associated with chronic intravascular haemolysis and thromboembolic events. Although PNH is a non-neoplastic clonal disorder, half of patients with acquired aplastic anemia (AA) develop PNH clones and it has long been associated to mielodysplastic syndromes (MDS).

A 52-year-old woman was admitted into emergency department referred metrorrhagia, asthenia and fatigue, with initial suspect of acute leukemia. Admission laboratory exams showed 7.3 g/dL Hb, 2.48x106 Red Blood cells, 2.04x103 White Blood cells (6.6% Neutrophils – 79.5 fl MCV, 89.8% Lymphocytes, 0.4% Monocytes, 0.9% Eosinophils, 0.1% Basophils), 39x103 Platelets. Upon discharge, the patient has been diagnosed with severe aplastic anemia and was admitted as outpatient to Hematology Unit. Flow cytometric assay displayed a lymphoid component equal to 79% and 68%, both in peripheral blood and bone marrow respectivetly, showing no alterations on B, T and NK lymphocytes. In contrast, alterations in maturation curve of granulocyte population was detected. A bone marrow biopsy revealed about 80% of plasma cells and lymphocytes, hemophagocytosis phenoma and reduced cellularity (cells<10%). Furthermore, molecular biology analysis were performed to investigate a panel of mutation and chromosomal traslocation such as Bcr/Abl, AM1/ETO, CBFB/MYH11, MLL/AF9, MLL/AF6, PML/RARα, A-B-D mutation on NPM gene. The results showed that patient didn't carry any of these mutations. Based on the investigation performed during admission PNH was suspected, so cytometric analysis was performed on peripheral blood demonstrating the presence of cells defective in glycophosphatidylinositol (GPI)-linked molecules in granulocytes population. The patient was treated with anti-thymocyte globulin (ATGAM), cyclosporine and revolade. The patient is currently under investigation in order to monitorate the therapy induction.

In conclusion, the case highlights the importance of flow cytometry as an helpful tool, given the complex pathophysiology of PNH disease.

NORMALIZED BLAST COUNT: A FLOW CYTOMETRY STANDARDIZED INDEX FOR QUICK EVALUATION OF NORMAL RESIDUAL HEMOPOIESIS

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Background. Myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) are clonal malignant myeloproliferative disorders characterized by increased proliferation of poorly differentiated myeloid neoplastic cells that progressively infiltrate the bone marrow (BM) ultimately leading to exhaustion of residual normal hematopoiesis and peripheral blood (PB) pancytopenia. Evaluation of normal hematopoiesis and leukemic cell infiltration of BM is currently performed by optical microscopy carried out by physicians, with operator-dependent variabilities. Flow cytometry is more sensitive and specific in distinguishing normal myeloid cells from neoplastic counterpart even just using linear parameters and CD45 expression. We have showed that a simple integrated approach using blast counts by flow cytometry, FLT3 mutational status, and WT1 expression levels could be a useful tool for a better prognostic definition in AML and MDS. Normalized blast count (NBC) could be an easy standardized prognostic index allowing inter-laboratory data comparison and quick evaluation of normal residual hematopoiesis.
Materials and Methods. A total of 88 patients with AML (N = 35) or MDS (high-risk, N = 7; low-risk, N = 46) were included in this retrospective study after informed consent obtained in accordance with the Declaration of Helsinki. A group of healthy subjects (N = 20) was used as a control. Heparinized or ethylenediaminetetraacetic acid (EDTA) peripheral blood (PB) specimens were directly stained with the following antibodies: for PB immunophenotyping, CD56, CD45, CD34, CD19, CD11b, CD3, CD8, CD71, CD33, CD16, SmIg-kappa, and SmIg-lambda; for BM immunophenotyping, CD3, CD7, CD5, CD19, CD34, CD16, CD11b, CD13, CD14, CD56, CD45, CD33, HLA-DR, CD117, SmIg-kappa, and SmIg-lambda. CD45dim blast phenotype was further studied for CD19, CD20, CD34, CD56, CD5, CD117, CD33, CD16, CD11b, CD36, CD13, HLA-DR, CD64, CD4, CD5, CD7, CD14, CD10, CD15, CD11a, CD11c, CD45RA, CD45RO, CD61, CD42b, TdT, and MPO expression CD45; CD4; CD8; CD3; CD56; CD19; CD5; CD23; CD10; CD11c; CD20; CD103; CD38; CD49d; SmIg-Kappa; and SmIg (all from Beckman Coulter, Milan, Italy). Samples were incubated for 20 minutes at 4°C, and then 3 mL of red blood cell lysis buffer was added, samples incubated for 15 min at room temperature, centrifuged, and cell pellets resuspended in 500 μL of PBS. Sample acquisition was performed on a ten-color three-laser Beckman Coulter Navios Flow Cytometer (Beckman Coulter). Post-acquisition analysis was carried out using Navios tetra or Kaluza Analysis Flow Cytometry software v2.1.1 (Beckman Coulter). Instrument daily quality control was carried out using Calibrite Beads or Flow-Check Pro Fluorospheres (Beckman Coulter), and external quality control by UK NEQAS for Leucocyte Immunophenotyping. Samples were run using the same PMT voltages, and at least 1 million events were recorded. NBC was calculated as following: NBC = (%CD34+ cells + %immature cells + %blasts)/%granulocytes, using frequencies measured by flow cytometry, and a cut-off of 0.5 was employed for definition of increased (> 0.5) or normal (<0.5) levels.
Results. NBC values were calculated for each subject and compared between groups by ANOVA and Uncorrected Fisher's LSD for multiple comparisons tests. NBC values were significantly higher in AML compared to low-risk MDS (mean+SD, 8.48+21.3 vs 0.07+0.06, respectively; P = 0.0022) and healthy subjects (mean+SD, 0.03+0.03; P = 0.0135). No significant differences were observed between high-risk and low-risk MDS or healthy subjects.
Conclusions. Our results showed diagnostic utility of NBC for a standardized and fast evaluation of normal residual hematopoiesis in AML patients.

EVALUATION OF WBC CONCENTRATION IN LEUKOREDUCED PRODUCTS BY FLOW CYTOMETRY WITH LEUKOSURE ENUMERATION KIT

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Objectives
The presence of white blood cells (WBCs) in blood products for transfusion can cause adverse effects in patients; according to European and Dutch requirements, at least 90% of the tested WBC-reduced blood products should contain fewer than 1 x 106 WBCs per product, which corresponds with 3.3 WBCs per μl for a 300 ml blood product. The LeukoSure Enumeration Kit is configured to utilize the sensitivity of flow cytometry to enumerate residual leukocytes at the levels necessary to ensure the quality control of leukoreduced blood products.

Materials and methods
From January 2022 to March 2023 we collected and processed 340 samples of pools of platelets (PLTs) and 103 samples of red blood cells (RBCs). In the test tube, 100 μl of blood product and 100 μl of Lyse Reagent were added. After mixing, 500 μl of Stain Reagent were added, the samples were mixed again and incubated for 15 minutes at room temperature in the dark. Finally, 100 μl of Fluorospheres were added. The samples were measured on the FC 500 flow cytometer and for each sample a fixed number of 10.000 beads events was counted. Leuko-Trol Platelet Control Cells, which contain mammalian platelets and human leukocytes in a plasma-like fluid, were employed as low (2.8 ± 1.5 WBC/μl) and high (21.5 ± 5.4 WBC/μl) positive controls.

Results
337 out of 340 of pools of PLTs (99%) (average weight 448.5g and average pH 6.6) showed a WBCs count below 3.3 cells/μl and 3 out of 340 (1%) have been eliminated and not used for therapeutic purposes because WBCs count was found to be higher than 3.3 cells/μl. 100 out of 103 of RBCs (97%) had a WBCs count below 3.3 cells/μl, 2 out of 103 (2%) showed a value included from 3.3 to 5.0 cells/μl and in 1 out of 103 (1%) the WBCs count was found higher than 5.0 cells/μl. For both the WBC-reduced blood products, the known concentration of Fluorospheres was of 1010 beads/μl.

Conclusions
As shown, 99% of the routinely produced pools of PLTs and 97% of RBCs were WBC-reduced which amply conforms to the requirement that at least 90% of the products should contain fewer than 3.3 WBCs/μl. These findings suggest that the process of WBC reduction is adequate and robust and that the LeukoSure Enumeration Kit flow cytometric method represents a suitable and reliable tool for quality control of leukoreruced products.

DNT, DPT AND NKT-LIKE CELLS CYTOFLUORIMETRIC IDENTIFICATION IN B-CHRONIC LYMPHOCYTIC LEUKEMIA

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Double Negative T (DNT), Double Positive T (DPT) and Natural Killer T (NKT) cells play a relevant role in tumors.
Fifty patients with untreated B-CLL and classified in three prognostic groups (A Low-, B Intermediate- and C High-risk) and 38 healthy donors were identified in this study.
Total T, B, NK, NKT-like, DNT and DPT cells were evaluated in peripheral blood by flow cytometry. The percentages of each lymphocyte subpopulation showed a reduction from the values of controls due to the increase in the percentage of CD19+ neoplastic B cells in CLL patients. However, through the comparison of all small T subpopulations stratified for their respective prognostic group versus healthy subjects, an increase in DNT and NKT absolute values was found, while any significant changes were observed for DPT cells. The expansion of NKT-like cells in group A (that did not significantly diminish as percentages) could be due to the significant expansion of WBC, contrary to NKT-like cells in group B and C, that was significantly reduced respect to control values as percentage value. Furthermore, the study showed that a significant correlation of absolute values of NKT-like cells compared to B cells was observed only in prognostic B group. No significant correlation of B cells to DNT or DPT cells (on total patients or by each prognostic grop) was found.
A significant increase in total T lymphocytes in patients with B-CLL has previously been reported in the literature. Based on previous data that reported the expansion of Tc and Th cells in B-CLL, we analyzed the contribution of all T subpopulations on T cells increase, focusing attention on DNT, DPT and NKT-like subsets. So, this study demonstrated that absolute CD3+ T cell counts were increased in B-CLL patients largely due to expansion of Tc, Th and DNT, regardless of prognosis group. In particular, the best correlations were observed in groups B and C, probably due to the staging. NKT-like and DPT marginally caused the rise of absolute CD3+ T cell counts; in particular, only in group B correlated positively with the increase in T cells, not in groups A and C. The same statistical analysis was performed in healthy donors and the correlations were higher for CD8 and CD4 cells, similar for DPT and NKT-like cells, but curiously lower for DNT cells. The counterevidence in healthy subjects and the significant correlation in patients with B-CLL could suggest that the increase in DNT is due to an immune response of DNT cells to the disease. Analyzing the variations exclusively between prognostic groups there were no significant variations for the subpopulations of interest.
These results could suggest that the monitoring of these cell numbers may provide helpful information for evaluating disease activity. These results supported the concept that active B-CLL immune surveillance of DNT cells is independent of disease staging. In contrast, DPT and NKT-like cells appear significantly only as the disease progresses.

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FLOW CYTOMETRY ANALYSIS APPLICATION IN RESIDUAL DISEASE MONITORING OF A CASE OF ACUTE MYELOID LEUKEMIA EVOLVING FROM VEXAS MYELODYSPLASTIC SYNDROME

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VEXAS (Vacuoles, E1 enzyme, X linked, Autoinflammatory, Somatic) syndrome is a recently described disease caused by somatic mutations in the ubiquitin-like modifier activating enzyme 1 (UBA1) gene. It is characterised by overlapping variable autoinflammatory clinical manifestations and a typical morphological finding is the presence of cytoplasmic vacuoles in both erythroid and myeloid precursors in bone marrow. It is frequently associated with diagnosis of myelodysplastic syndromes (MDS). Despite no standard treatment has been established yet, azacitidine and allogeneic hematopoietic stem cell transplantation (allo-HSCT) are promising therapeutic options, but the indications for these treatments are not easily applicable to all patients. Here we report the first case of VEXAS Syndrome evolving to AML.
A 59-year old patient was indeed initially diagnosed with low-risk MDS with multilineage dysplasia and systemic autoinflammatory symptoms, that nearly one year after first diagnosis evolved to Acute Myeloid Leukaemia (AML). The patient was firstly treated with azacytidine and venetoclax (Aza-ven) that was subsequently stopped due to impaired performance status. Afterwards, due to disease relapse, a 3+7 schedule was started in order to perform allo-HSCT. Importantly, flow cytometric analysis allowed rapid diagnosis and also helped for monitoring residual disease after treatment. Longitudinal monitoring of UBA1 in droplet digital PCR was also performed for disease monitoring.
Here we summarize bone marrow evaluations with flow cytometry and UBA1 analysis at different timepoints:
a) At diagnosis (January 2021) morphology showed dysplastic features and vacuolization in the erythroid and granulopoietic lineage with 3% of blasts. CD34+CD45+ cells in flow cytometry were 1.8%; UBA1 mutation was found positive with a VAF of 49%
b) Progression to AML (March 2022): 24% agranular blasts at cytological examination. In flow cytometry 16% blasts expressing CD45dim/CD34+/CD117+/HLA-DR.+/CD13+/CD33+ (UBA1 mutation VAF of 68%)
c) After the first cycle of Aza-ven (May 2022) morphological remission was observed (4% blasts) with 2% CD34+CD45+ cells in flow cytometry (UBA1 not evaluated)
d) After the second cycle of Aza-ven (June 2022) no blasts on the cytological smear, CD34+CD45+ cells were 0.9%, (UBA1 mutation VAF of 1,39%)
e) Relapse (September 2022) of both autoinflammatory symptoms and leukemia with 14% blasts in BM and 2% CD34+CD45+ cells in flow cytometry (VAF for UBA1 increased to 23.9%)
f) Post 3+7 induction treatment (November 2022) bone marrow examination showed no blasts and CD34+CD45+ <1% with UBA1 mutation being nearly
undetectable (VAF of 0.07%)
To our knowledge, this is the first case of AML transformation in the context of VEXAS syndrome, and we report the use of flow cytometric analysis as a valid tool of residual disease monitoring in this context, to be coupled with other techniques such as drop digital PCR for UBA1 monitoring.

Immunology

A NOVEL BASOPHIL ACTIVATION TEST FOR DIAGNOSIS OF IMMEDIATE HYPERSENSITIVITY REACTIONS TO TAXANE

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Taxane (Tx) and platinum (Pl)-based chemotherapy (CT) has represented the key component treatment of gynaecological and breast cancer over the past 20 years. However, Tx and Pl-induced immediate hypersensitivity reactions (iHSRs) have become increasingly frequent in clinical practice, because of longer survival of cancer patients and increased chance to administer more CT lines and more cycles for each line. Yet, several personalized premedication/desensitization strategies allow patients to continue CT after iHSR occurrence. Choice of the most appropriate desensitization strategy is generally based on iHSR grade and on results of skin testing and laboratory tests, i.e., serum specific IgE assay and Basophil Activation Test (BAT). However, in the case of Tx neither serum specific IgE assays nor BAT are commercially available. Thus, development of drug-specific in vitro allergy testing is urgently needed for accurate diagnosis of iHSR and treatment choice and for a thorough definition of immune and non-immune mechanisms involved in iHSR to Tx. In the present study, we optimized a flow-assisted BAT aimed at supporting accurate diagnosis of allergy to Tx and at integrating information for a personalized and effective iHSR management program. To this end, we have introduced a novel BAT acceptance criterion aimed at checking for basophil (Ba) viability in the BAT after in vitro exposure to Tx, in order to keep under control Ba performance and prevent false negative test results. Twenty-eight patients were enrolled in the study (17 had iHSR to Tx, and 11 were tolerant to Tx). Ba were identified as CD45+CCR3+CRTH2+CD3- cells with peculiar SSC properties and their degranulation measured as increase in CD203c fluorescence intensity and CD63 expression. A significant association between increase in both the CD63 and CD203 degranulation markers and iHSR to Tx was found. An algorithm was created, which included both degranulation markers, and served for establishing a cutoff value that correctly recognized patients with iHSR to Tx (cutoff value≥0.024) from tolerant patients. Because Ba in the BAT can degranulate via either IgE or non-IgE mediated stimuli, we added ibrutinib, a known inhibitor of the FcεRI-dependent Ba degranulation, in our BAT to provide in vitro evidence on the causative mechanism of Ba degranulation. By this approach, we could recognize when Ba degranulation was dependent on IgE cross-linking in a selected set of BAT-positive patients.

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BENRALIZUMAB AFFECTS NK CELL MATURATION AND PROLIFERATION IN SEVERE ASTHMATIC PATIENTS

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Introduction: Benralizumab is an anti-interleukin-5 receptor α monoclonal antibody with antieosinophilic activity. A particular mechanism of action of this drug is determined by its afucosylated constant fragment (Fc) that binds FcγRIIIa receptors on the membrane of natural killer (NK) cells.
After this interaction, NK cells activate antigen-dependent cell cytotoxicity (ADCC) through the release of proapoptotic proteins, leading to apoptosis of eosinophils.
Objectives and Methods: Multiparametric flow cytometry of NK and T cell subsets was performed in severe asthmatic (SA) patients (n=10), before (T0) and after 1 (T1) and 6 (T6) months of benralizumab, and compared with mild and moderate (M/M) asthmatic patients (n=9) and healthy controls (HC) (n=15). Serum cytokines levels were detected through multiplex assay. Proliferation assay through flow cytometry was performed at baseline and during the follow-up of therapy.
Results: At baseline, SA patients showed lower percentages of mature CD56dimCD16br NK when compared with HC and M/M (65±22,2 in HC vs 65,3±20,3 in M/M vs 25±23,9 in SA respectively; p=0,0026). After benralizumab administration, a shift of NK cell phenotypes towards maturity and towards the release of cytotoxic mediator IFN-γ (0.52±0.3 at T0, 0.72±0.52 at T1 and 1.15±0.6 pg/ml at T6; p=0.0114) were observed. Decrease percentages of T Effector memory were reported for both CD4+ cells (61,1±22,6 at T0 and 51,2±22,4 at T6) and CD8+ cells (49,6±9,6 at T0 and 37,7±18,5 at T6) after 6 months of therapy. An indirect correlation between the proliferation state of NK cells and steroid-sparing (r=-0.94 p=0.005) was reported.
Conclusions: Together these data contribute to our understanding of the mechanisms of action of benralizumab in the resolution of inflammation in SA patients. The activation and maturation of NK cells promoted by benralizumab allow the depletion of eosinophils and mediate T cell development.

DEVELOPMENT OF A NOVEL ROBUST FLOW CYTOMETRY APPROACH FOR IDENTIFICATION OF PERIPHERAL MYELOID-DERIVED SUPPRESSOR CELLS OF GRANULOCYTE ORIGIN IN CANCER PATIENTS

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Myeloid-derived suppressor cells (MDSC) are a heterogeneous population that play a prominent role in the establishment of the immunosuppressive tumor microenvironment. High peripheral MDSC counts have been associated with great tumor burden, poor response to immunotherapy and poor survival in cancer patients. MDSC can be subdivided into two main cell subsets distinguished by phenotype and activity: monocyte-MDSC and polymorphonuclear (PMN)-MDSC. Peripheral PMN-MDSC are abundant in cancer patients and their frequency correlates with intratumoral PMN-MDSC. Thus, blood represents an easy-accessible and viable source for measuring PMN-MDSC and the minimal phenotypic signature CD45+CD15+CD11b+Lox-1high (aka PMN-MDSC phenotypic backbone), together with PMN-like light scattering properties, should allow distinction between PMN-MDSC and PMN in whole blood (WB). However, WB PMN are exquisitely sensitive to changes in processing methods and certain pre-analytical variables, including time elapsed between blood collection and testing and anticoagulant types, alter the PMN activation status. Because activated PMN (aPMN) are phenotypically and morphologically indistinguishable from PMN-MDSC when using the PMN-MDSC phenotypic backbone, more research is urgently needed to solve this issue, especially as far as clinical immunomonitoring studies are concerned. Thus, in this study we search for a new PMN-MDSC-specific phenotype allowing for recognition of genuine PMN-MDSC from aPMN in WB. To this end, selected antibodies to myeloid cell-associated surface receptors were added to the PMN-MDSC phenotypic backbone and tested for ability to distinguish genuine PMN-MDSC from aPMN under diverse pre-analytical conditions. Results showed that the CD45+CD15+Lox-1highCD11bdimCD66bdim/bright phenotype permitted stable enumeration of PMN-MDSC, irrespective of diverse WB processing conditions. In order to confirm reliability of this novel PMN-MDSC phenotype, we tested these cells for functional markers and suppressive function by flow-cytometry and showed that they express high intracellular arginase-1 levels and are endowed with ability to suppress T cell proliferation. Thus, we provided evidence for PMN-MDSC in WB to be unequivocally defined by this novel immunophenotypic signature. We then evaluated CD45+CD15+Lox-1highCD11bdimCD66bdim/bright PMN-MDSC frequency in the WB of ovarian, cervical, breast and non-small cell lung (NSCL) carcinoma patients, ahead of any treatment. All patients presented with higher WB PMN-MDSC levels than healthy subjects. Interestingly, the highest levels were found in NSCL carcinoma patients.

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A MULTICENTER STUDY OF THE EFFECTS OF INTERSTITIAL LUNG DISEASES ON THE ALVEOLAR EXTRACELLULAR VESICLE PROFILE

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Diagnosis of interstitial lung diseases (ILD) is difficult to perform. Extracellular vesicles (EVs) facilitate cell-to-cell communication, and they are released by a variety of cells. Our goal aimed to investigate EVs markers in bronchoalveolar lavage (BAL) from idiopathic pulmonary fibrosis (IPF), sarcoidosis and hypersensitivity pneumonitis (HP) cohorts.

ILD patients followed at Siena, Barcelona and Foggia University Hospitals were enrolled. BAL supernatants were used to isolate the EVs. They were characterized by flow cytometry assay through MACSPlex Exsome KIT

Majority of alveolar EVs markers were related to the fibrotic damage. CD56, CD105, CD142, CD31 and CD49e were exclusively expressed by alveolar samples from IPF patients, while HP showed only CD86 and CD24. Some EVs markers were in common between HP and sarcoidosis (CD11c, CD1c, CD209, CD4, CD40, CD44, CD8). Principal Component Analysis distinguished the three groups based on EVs markers with total variance of 60.08%

This study has demonstrated the validity of the flow cytometric method to phenotype and characterize EVs surface markers in BAL samples. The two granulomatous disease, sarcoidosis and HP cohorts shared alveolar EVs markers not revealed in IPF patients. Our findings demonstrated the viability of the alveolar compartment allowing to identify lung-specific markers for IPF and HP.

IMMUNOPHENOTYPIC CHARACTERIZATION OF PERIPHERAL BLOOD-DERIVED B LYMPHOCYTES OF PATIENTS WITH SYSTEMIC LUPUS ERYTHEMATOSUS DURING B-CELL TARGETED THERAPY WITH ANTI-BLYS

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Background and aims.
BLyS inhibition following Belimumab therapy is associated with circulating B-cell subsets and short-lived plasmacells reduction. The aim of this study was to characterize the B cell phenotype in Systemic Lupus Erythematosus at baseline and after anti-BlyS treatment.
Methods.
Fifty-four active SLE patients (49 females, mean age 40.6±13.2 years, disease duration 12.3±9.0 years, SLEDAI-2K 6.6±3.1) who received Belimumab were enrolled. Phenotyping of peripheral blood B cells (using as phenotypic markers IgD, CD38 and CD27) was performed at six (T6) and twelve (T12) months by flow cytometry.
Results.
In the whole SLE cohort a reduction of CD19pos [T0: 11.1±6.1% vs T6: 6.4±3.4%, p<0.01; T12: 4.2±3.4%, p<0.01] and CD19posIgDposCD27neg [T0: 55.8±28.7% vs T6: 34.9±22.2%, p<0.01; T12: 30.0±19.4%; p=0.04] and an increase of CD19posIgDnegCD27pos [T0: 21.0±20.2% vs T6: 37.5±21.4%, p<0.01; T12: 42.2±21.7%, p=0.02] after therapy was observed. Stratifying patients based on organ involvement, a reduction of CD19pos [T0: 10.7±4.6% vs T6: 6.8±2.4%, p=0.03; T12: 4.5±3.5%, p=0.03] and CD19posIgDposCD27neg [T0: 61.0±24.6% vs T6: 38.9±17.5%, p<0.01; T12: 36.9±16.0%, p=0.03] in patients with mild organ involvement and an increase of CD19posIgDnegCD27posin both subgroups [(severe SLE T0: 24.1±25.0% vs T6: 44.9±27.4%, p=0.01) (mild SLE T0: 18.9±18.3 vs T6: 31.2±12.7%, p<0.01)] was found. Evaluating the B cell subsets according to the treatment response, a reduction of CD19posIgDposCD27neg at T6 [(responders T0: 55.4±29.3 vs T6: 32.3±19.9, p<0.01) (no-responders T0: 63.1±41.3% vs T6: 41.4±33.5%, p=0.05)] and an increase of CD19posIgDnegCD27pos [(responders T0: 22.4±21.2% vs T6: 39.6±19.4%, p<0.01) (no-responders T0: 20.6±26.1% vs T6: 38.6±35.3%, p<0.05) was observed in both groups. ROC curve analysis of IgDnegCD27possubset identified a cut-off of 9.94% [AUC(95% CIs:0.761: (0.566-0.957),p=0.023) associated with response at T6. Moreover, having an IgDnegCD27pos rate ≥9.94% [OR:4.5(95%CIs:0.9-17.2)] and the presence of anti-dsDNA antibodies at baseline [OR:5.2(95%CIs:1.2-22.1)], identified patients who achieved early response within T6 from therapy initiation.
Conclusions.
Analysing B cells subsets, a different immunophenotype emerged in SLE non-responder patients compared to responder patients, probably configuring a B-cell phenotype more resistant to anti-Blys therapy in non-responders. Moreover, baseline immunological features and IgDnegCD27posB cell subset rate could be novel putative biomarkers of response to anti-BLyS therapy in SLE.

EFFECTS OF PROBIOTICS ON FUNCTIONAL ACTIVITIES OF THP-1-DERIVED MACROPHAGES IN COMBINATION WITH MORINGA OLEIFERA AQUEOUS EXTRACT

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Background

The emerging role of chronic, low-grade inflammation in the progression of diseases typical of modern society has prompted the scientific community to comprehend the influence of nutrition and dietary patterns on inflammation. The gastrointestinal tract can suffer the effects of chronic inflammation, leading to a persistent activation of the immune system.

Moringa oleifera Lam. (MO), commonly used in African traditional medicine, is widely known for its high content in nutrients and bioactive compounds, that have been characterized in all parts of the plant, especially in leaves and seeds. Many studies have confirmed MO pharmacological properties on several metabolism-related diseases, in particular, MO has been proven to have anti-inflammatory, anticancer, and immunomodulatory properties.

Lactic acid bacteria (LAB) are recognised as common inhabitants of the human gastrointestinal tract, and have received attention in recent years due to their postulated health-promoting effects. There is evidence that probiotic bacteria can modulate the host's immune response.

Materials and Methods

Cell cultures and differentiation. The human monocytic cell line THP-1 was induced towards differentiation with PMA at a concentration of 20 ng/mL.

MOES preparation. The extract of Moringa oleifera seeds (MOES) was prepared according to an African traditional method and administered to cells at a concentration of 2.5 mg/ml.

Probiotic cultures. Lyophilized cultures of Lactobacillus plantarum TJA7 were provided by Probionova SA (Basel, Switzerland), purified by subsequent subcultures and administered to cells at a Multiplicity Of Infection (MOI) of 500 bacteria : 1 cell.

Cytokine staining. Cytokine expression was evaluated by harvesting 0.5 x 106 cells for each treatment at different time points (0, 3, 6, 12, and 24 hours), washed with PBS, permeabilized and stained with 0.5μl of monoclonal antibodies specific for the following cytokines: TNF-α, IL-6, IL-8, IL-1β, IL-10 and surface antigens: CD16/CD11b and CD36.

Results

The effect of the co-treatment of MOES and of L. plantarum (LP) on cytokines production in THP-1-derived macrophages was observed at 0, 3, 6, 12 and 24 hours, alone or in combination with LPS stimulus (20ng/mL). Without LPS, differentiated THP-1 cells showed changes in the percentage of cells producing different cytokines, suggesting a stronger and synergistic effect as compared to single treatments. The percentage of TNF-α positive cells stimulated with LPS and treated LP + MOES was reduced respect to LPS alone, that instead present a peak after 6 hours. The percentage of IL-8 positive cells was lower in MOES+LP treatment and MOES alone respect to the control after 12 hours. Also the percentage of IL-10 positive cells appears to increase after 3 hours respect to control. When in presence of LPS, this treatment induces a reduction of CD36+ cells in every time point.

Conclusion

The results suggest that cells treated with MOES and LP in inflammatory conditions do not undergo a further inflammatory state but tend to upregulate specific cytokines to recruit other cell populations in the site of inflammation and enhance the capability of macrophage to recognize antigens through CD36.

AN INNOVATIVE FLOW CYTOMETRY TEST FOR EARLY DIAGNOSIS OF INVASIVE BACTERIAL AND VIRAL INFECTION

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Early diagnosis and immediate treatment of invasive bacterial and viral infection reduce the risk of death. Bacteria culture tests and molecular tests are the gold standard for differential diagnosis of bacterial and viral infection. However, they may require several days to be performed, thereby reducing the clinical utility, may be invasive (e.g,bronchoalveolar lavages) and a substantial proportion of patients even with severe sepsis are culture negative. Present flow cytometry (FC) test optimizes and simplifies previous FC tests by minimizing the influence of variables inherent in FC analysis (instrument and/or of reagent performance, operator experience, subjective analysis), is easy-to-perform, quick (execution time ~40 minutes) and requires only 50μl of venous blood making it applicable to paediatric patients. The test measures the increase in Fcγ receptor type 1 (CD64) expression on polymorphonuclear granulocytes (PMN) as indicator of bacterial infection and the increase of Siglec-1 (CD169) expression on monocytes (MO) as indicator of viral infection. The test also measures HLA-DR expression reduction and type 2 interleukin 1 receptor (IL-1R2) expression increase in MO as indicators of immunocompromised status. We analysed 78 patients (age: 31-88 yrs, median 67 yrs; M/F ratio 1.8) from emergency room and hospitalization wards, in which infection was diagnosed clinically by the managing physicians. Seventy-six percent of patients had bacterial infection, 11% had bacterial/viral co-infection and 13% had viral infection with no evidence of bacterial co-infection. Presence of bacteria and/or viruses was sought with standard microbiological tests on various bodily samples (blood, urine, sputum, bronchoalveolar lavages, pharyngeal swabs, etc.). Age and gender matched health care professionals (n=15) with no evidence of infection were included as comparison. A significant increase in CD64 expression was present in >98% of patients with bacterial infection, irrespective of concomitant viral co-infection; a significant increase in CD169 expression was seen in >98% of patients with viral infection, irrespective of bacterial co-infection. Three of the 4 patients with reduced HLA-DR expression and concomitant IL-1R2 increased expression died. CONs: 1) The test does not provide indications about the responsible bacterial or viral species and, in the case of bacterial infections, about the sensitivity to the different antibiotics. 2) Modulations of different markers may potentially reflect the presence of cytokines not induced by pathogens. This hypothesis is currently under study.

EVALUATION OF BASOPHILS SUBSETS IN PATIENTS WITH SEVERE EOSINOPHILIC ASTHMA AND THEIR RESPONSE TO THE ANTI-IL5R MONOCLONAL ANTIBODY BENRALIZUMAB

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Basophils are the least common granulocytes, representing less than 1% of peripheral blood leukocytes in both mice and humans. These cells share some features with tissue-resident mast cells even if they differ in several aspects. Of great interest is the correlation of basophils and eosinophils in asthmatics. Suzuki et al demonstrated a higher sensitivity, specificity, positive predictive value and negative predictive value of sputum basophil counts for the discrimination of an eosinophilic asthma phenotype than blood eosinophil count and exhaled nitric oxide (1). Although the contribution of basophils to the pathogenesis of asthma in humans remains poorly understood, studies have identified a high number of basophils in both post-mortem lung tissue and bronchial biopsies of asthmatic patients (2-3). Basophils are increased in the sputum of eosinophilic asthmatics compared to non-eosinophilic asthma phenotypes but, interestingly, basophils are increased in the sputum of both allergic and non-allergic eosinophilic asthmatic patients (8-9). Basophils have a multifaceted role in several immunological processes in asthma. Basophil-derived IL-4 plays a role in the activation of both naïve T helper cells and innate lymphoid cells (ILC) toward a type 2 response (4). Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response (6). Basophil-derived IL-4 has been shown to regulate the infiltration of eosinophils and it could be speculated that early basophil migration into the lungs during exacerbations might be important for subsequent infiltration of eosinophils into airways. Understanding the factors that regulate basophil trafficking and activation might lead to better clarify their role in asthma pathogenesis.
Two phenotypically distinct basophil populations in the peripheral blood have been identified: IL-3-elicited basophils which degranulate in response to IgE-mediated crosslinking, and the other TSLP-elicited that exhibit a limited ability to degranulate after IgE-mediated crosslinking, responding robustly to stimulation with IL-3, IL-18 or IL-33 (10). Collectively, these studies highlight that IL-3-elicited and TSLP-elicited basophils differentially respond to IgE-mediated activation and can be further distinguished by their response to a variety of cytokines.
We evaluated, by flow cytometry, the different subpopulations of circulating basophils in patients with severe eosinophilic asthma, treated with Benralizumab (30 mg/4 weeks for 3 times and then 30 mg/8 weeks) at baseline and after 6 months of therapy. In particular, plasma samples were collected from each patient and analyzed by flow cytometry to characterize basophils surface receptors (IL-3R, IL-5R, TSLP-R, IL-25R, IL-33R, and FcεRI) expression at the baseline and after 6 months of treatment.
We will report in detail the gating strategy and the preliminary results obtained.
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IN DEPTH CHARACTERIZATION OF HUMAN CMV-DRIVEN ADAPTIVE NK CELLS THROUGH MASS SPECTROMETRY AND FLOW CYTOMETRY ANALYSES

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Human Natural killer (NK) cells are a population of innate lymphocytes, usually short-lived and rapidly responding against viral infections and tumors. However, upon cytomegalovirus (CMV) infection, NK cells reveal unexpected adaptive traits typical of T cells, such as enhanced effector function and longevity. CMV-driven adaptive NKs show high NKG2C (i.e. a HLA-E-specific activating receptor) levels, a mature phenotype (CD57+KIR+NKG2A-), epigenetic modifications and could display anti-tumor properties. In view of their longevity and specialized functional features, adaptive NK cells are suitable candidates in cellular immunotherapy.
Adaptive NK cells have been investigated so far mainly through cytofluorimetric approaches and transcriptomic studies. Thus, to better characterize and possibly identify novel functional pathways associated to this NK cell subset, we have set up a mass spectrometry proteome analysis on FACS-sorted adaptive NK cell subsets isolated from six CMV-seropositive healthy donors. In particular we compared the proteomic signature of adaptive NKG2C+CD57+ NK cells with conventional NKG2C-CD57- NK cells, i.e. more immature NK cells lacking CMV-driven imprinting.
We identified 4618 proteins from NK cell subsets lysates, representing the largest protein catalogue reported so far for NK cells, and observed a sharply different proteome profile in adaptive compared to conventional NK cells. Indeed, we found 165 proteins that were significantly more expressed and 263 proteins that were significantly down-regulated on NKG2C+CD57+ NK cells as compared to NKG2C-CD57- NK cells. The different proteome profile shown by adaptive NK cells confirmed previous data obtained by cytofluorimetric studies, such as higher expression of LILRB1 and lower expression of CD161. On the other hand, our proteome analysis confirmed at the protein level data from transcriptomic studies, such as higher expression of the anti-apoptotic protein Bcl-2, possibly responsible for adaptive NK cell longevity, and of the signaling protein CD3e. Based on the hints from mass spectrometry data, we further investigated the most significant differentially expressed proteins through intracellular flow cytometry to validate our results in a larger donors cohort and to explore in detail NK cell subsets not included in the proteomic analysis. We could thus reveal that high Bcl-2 expression mainly characterizes NKG2C+ NK cells including not only adaptive NKG2C+CD57+ but also the less differentiated NKG2C+CD57- NK cells. In addition, we confirmed that cytoplasmic CD3e expression marks adaptive NKG2C+CD57+ NK cells highlighting the phenotypic proximity that this NK cell subset displays with T lymphocytes.
Thus, mass spectrometry proteome profiling of adaptive NK cells, combined to cytofluorimetric analyses, has provided further insights in their biology and revealed novel features that could be relevant also to better harness this NK cell subset for immunotherapeutic purposes.

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BIOMIMETIC PROTEOLIPID LEUCOSOME VESICLE CHARACTERIZATION BY FLOW CYTOMETRY IMMUNOPHENOTYPING

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ABackground. Novel platforms for drug delivery are required to highly increase molecule uptake by specific targeted cells simultaneously reducing off-target effects and to enhance drug efficiency. Liposomes consist of an aqueous volume entrapped by one or more bilayers formed by both natural and/or synthetic lipids. Indeed, cationic Liposomes, sterically stabilized, exhibit high affinity to cell membranes and can be used to deliver exogenous material intracellularly via cell membrane fusion. Liposomes are promising biocompatible vesicular carriers, also because of their amphipathic nature allowing encapsulation of a wide range of molecules. Despite chemical functionalization, carrier clearance is not completely prevented, therefore novel biomimetic strategies, including membrane protein incorporation within liposome bilayer, are of high interest as promising drug delivery systems. In this study, we characterized liposomes functionalized with human membrane proteins and assembled using a microfluidic technology, by mixing phosphocholine-based phospholipids plus cholesterol. These biomimetic proteo-lipid vesicles (BPLVs) were characterized for several surface markers to confirm that BPLVs can successfully incorporate human surface membrane proteins and can correctly orient them through the synthetic lipid bilayer.
Materials and Methods. BPLV characterization was carried out by flow cytometry immunophenotyping. To avoid bead use for vesicle identification by linear parameters, vesicles were rhodamine-conjugated, and particle concentration was assessed using Nanosight, an instrument for Nanoparticle Tracking Analysis (NTA) (Malvern Instruments Ltd, Malvern, Worcestershire, UK). Afterwards, a total of 1x107 particles were resuspended in 100 μL of filtrated water and were stained with FLAER-FITC (), CD3-ECD, CD33-PC5.5, CD14-Alexa Fluor 750, CD45-Krome Orange, and HLA-DR-SuperNova 780 (all from Beckman Coulter). After 20 min incubation at room temperature, samples were directly resuspended in a final volume of 300 μL of filtrated water for acquisition. Samples were acquired on a DxFlex cytometer (Beckman Coulter), equipped with violet (405 nm), blue (488 nm), and red (633 nm) lasers. Instrument daily quality control was carried out using CytoFlex Daily QC Fluorospheres (Beckman Coulter). Post-acquisition analysis was performed using FlowJo software (v.10.8.1; BD Biosciences).
Results. For BPLV characterization, vesicles were first identifying by gating for rhodamine and CD3, hypothesizing that correctly formed BPLVs incorporated both rhodamine-conjugated lipids and human surface proteins, such as CD3. Next, CD3+Rhodamine+ vesicles were studied for: CD14, a monocyte marker; CD33, a granulocyte / monocyte marker; CD45, a pan-leukocyte marker; HLA-DR, an activation marker; and FLAER, a molecule that binds the GPI-anchor thus identifying all GPI-linked proteins and being a marker of the presence of post-translational-modified proteins on BPLVs. An unstained sample of rhodamine-conjugated particles was used as negative control for setting gate boundaries. Human surface proteins were highly enriched on BPLV surface and correctly positioned within the lipid layers. In particular, CD33, CD14, and GPI-linked proteins were highly present, while CD45 and HLA-DR were at low levels, likely because of donor-dependent T cell activation status.
Conclusions. BPLVs can be successfully characterized by multiparametric flow cytometry immunophenotyping avoiding beads usage adopting rhodamine-conjugated lipids. However, titration should be performed during protocol optimization to identifying optimal particle and antibody concentrations.

IDENTIFICATION OF A NOVEL CORD BLOOD NK CELL SUBPOPULATION EXPRESSING FUNCTIONAL PROGRAMMED DEATH RECEPTOR-1

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Background: Natural Killer cells (NKs) represent the innate counterpart of T lymphocytes and are characterized by a high anti-tumor and an anti-viral cytotoxic activity. Recently, it has been demonstrated that NKs can express PD-1 as an additional inhibitory receptor. Specifically, PD-1 was identified on a subpopulation of terminally differentiated NKs from healthy adults with previous HCMV infection. So far it is unknown whether PD-1 appears during NK-cell development and whether this process is directly or indirectly related to HCMV infection.
Methods: In this study, we analyzed the expression and function of PD-1 on Cord Blood derived NKs (CB-NKs) on a large cohort of newborns through multiparametric cytofluorimetric analysis.
Results: We identified PD-1 on CB-NKs in half the newborns analyzed. PD-1 was present on CD56dim NKs, and particularly abundant on CD56neg NKs, but only rarely present on CD56bright NKs. Importantly, unlike in adult healthy donors, in CB-NKs PD-1 is co-expressed not only with KIR, but also with NKG2A. PD-1 expression was independent of HCMV mother seropositivity and occurs in the absence of HCMV infection/reactivation during pregnancy. Notably, PD-1 expressed on CB-NKs was functional and mediated negative signals when triggered.
Conclusion: To our understanding, this study is the first to report PD-1 expression on CB derived NKs and its features in perinatal conditions. These data may prove important in selecting the most suitable CB derived NK cell population for the development of different immunotherapeutic treatments.

PHENOTYPIC AND FUNCTIONAL STUDY OF CD8+ TISSUE-RESIDENT MEMORY CELLS IN THE BONE MARROW OF NEW DIAGNOSTICATED MULTIPLE MYELOMA PATIENTS

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Non-circulating resident memory T lymphocytes (TRM) constitute a cells subset that permanently resides in the peripheral tissues, including bone marrow (BM), and acts as sentinel against pathogens. In addition, these cells constitutively express specific tissue-homing markers like CD103. In recent years, several studies have assessed the possible role of CD8+ TRM in the immune response against tumours.
Multiple Myeloma (MM) is the clonal expansion of malignant plasma cells that can release monoclonal immunoglobulins or their fractions. It is part of a broader spectrum of pathological conditions, including monoclonal gammopathy of undetermined significance (MGUS) and smouldering multiple myeloma (SM). The malignant plasma cells, as their physiological counterpart, have a tropism for the bone marrow trophic niches, representing the perfect environment for the proliferation of these cells.
Considering that the onset of MM is a multistep process, which results in the colonization of BM, we analyzed, by flow cytometry and functional experiments, the consistency, phenotype, and functional properties of the BM TRM in 26 patients with a new diagnosis of MM and five patients with SM to evaluate a possible role of these cells in the immune response against malignant plasma cells infiltrating the BM.
The ex vivo average frequency of MM CD8+ TRM gated as CD3+, CD8+, CD103+, and CD69+ was 0.6%. These cells showed mainly (77,8%) effector memory (EM) phenotype (CD45RA-, CCR7-) while the 10,1% showed terminally differentiated (TEMRA) phenotype (CD45RA+, CCR7-). In five patients with smouldering myeloma (SM), a pre-malignant disease of plasma cells, we detected a 0,2% average frequency of CD8+ TRM cells, with the vast majority displaying an EM (80,5%) phenotype.
To evaluate factors able to induce the expansion of these cells in vitro, we cultured BM-derived mononucleate cells (MC) in the presence of three different combinations of homeostatic cytokines, IL-15, IL-7 plus IL-15, and IL-7 plus IL-15 and TGF-β. Results yield an increase in the percentage of CD8+ TRM in all conditions tested, especially under the proliferative input of IL-15. Furthermore, adding PHA to the mix of three homeostatic cytokines caused a more pronounced expansion of CD8+ TRM cells.
To study the possible cytotoxic immune function of MM patients' BM CD8+ TRM cells, we also evaluated CD107a/b expression on their cell surface after stimulation with ionomycin and PMA. Results showed significant degranulation of BM CD8+ TRM cells, confirmed by the appreciable cytotoxic activity of ex vivo expanded CD8+ TRM against autologous myeloma cells in a Cr51 cytotoxicity assay.
This study highlights the presence of a small but reactive population of CD8+ TRM with a potential cytotoxic capacity in the BM of MM patients. However, further studies are needed to profoundly investigate the functional role of these cells against myeloma cells, also in the perspective of possible use in future therapeutic programs.
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SPATIAL PROFILING OF TUMOR-INFILTRATING IMMUNE CELL SUBPOPULATIONS IN CHILDHOOD EPENDYMOMA

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Immune-therapy is a strategy for targeting a variety of tumors and tumor microenvironment (TME) composition is often related to patients' response. Tumors are frequently described as immunologically "hot" or "cold". In "hot" tumors the TME is infiltrated by T cells, suggesting an ongoing immune response, while TME of "cold" tumors contains only few T cells, suggesting a weak immune response. Thus, classifying a tumor as "hot" or "cold" may predict immunotherapy efficacy. Additionally, spatial and temporal heterogeneity of cancer-cell clones with diverse molecular and biological characteristics can condition tumor ability to overcome microenvironmental constraints, such as growth factors and oxygen deprivation, acidic and/or hypoxic conditions, vascularization, immunological surveillance and exposure to drugs. Thus, understanding the complexity of the TME and of its role as main driver of tumor evolution is of outmost importance. Ependymomas (EPN) are the second most common malignant tumor of the Central Nervous System (CNS) in children. The mainstays of treatment consist in surgery and radiotherapy, chemotherapy being generally ineffective. Thus, novel therapeutic strategies such as immunotherapy are being explored for achieving long-term disease control. Studies focused on genomics, transcriptomics and methylomics have greatly expanded our understanding of the biology underlying childhood EPN and single-cell RNA-seq studies have revealed the heterogeneity of tumor and TME. These approaches, however, ignore key features of anatomical connections, including spatial arrangement. We performed TME spatial phenotyping in childhood EPN at diagnosis and at relapse to explore the main immune and tumor cell subpopulations, and their connections, using the Tyramide Signal Amplification-based Opal assay for the TME immunofluorescence staining. We used the following antibody panels: CD68/CD163 (M2 macrophages); CD68/CCL2 (M1 macrophages); CD68/CD44 (macrophages associated to tumor angiogenesis); CD68/CD163/Ki67 (activated M2 macrophages); CD68/CCL2/Ki-67 (activated M1 macrophages); CD8/CD4/FOXP3 (regulatory T cells); SP100/Ki67 (proliferating tumor cells); FOS/COL9A2 (neuronal tumor stem cells); CA9/CD44 (mesenchimal-like tumor cells), DNAF1/RSPH1 (astro-ependymal like tumor cells), AQP4/GFAP (ependymal like tumor cells). Preliminary data suggest the presence of a large spatial and temporal heterogeneity in the TME of childhood EPN that will probably provide useful information for future immunotherapy strategies.

MULTIPARAMETER FLOW CYTOMETRY FOR EVALUATION OF MOUSE AND HUMAN EOSINOPHILS AS POTENTIAL IMMUNE BIOMARKERS

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Eosinophils are a rare subset of bone marrow-derived granulocytes that play major roles in allergic diseases and parasitic infections. Eosinophils are also implicated in a variety of important biological functions, including host defense against bacterial and viral infections, and cancer. Circulating eosinophils are emerging as potential prognostic/predictive immune biomarkers in different clinical settings, including viral diseases like COVID-19, and cancer. Multiparameter flow cytometry (MFC) enables high-throughput profiling and classification of eosinophil subsets. Human blood eosinophils express a variety of cell surface receptors relevant to their identity, maturation, activation, apoptosis, adhesion, rolling, homing, and migration in tissues. They are defined as CD45+ leukocytes expressing CD294 (CRTH2), CD15 and the late differentiation marker Siglec-8, while lacking CD3, CD19, CD56, CD14 and CD16 lineage markers. In addition, their activation has been associated to upregulation of CD63, CD69 and CD11b, and downregulation of CD62L. Previous studies indicated that IL-33 induces potent activation of human and mouse eosinophils resulting in increased expression of CD69 and CD11b, the latter promoting adhesion and contact-dependent degranulation. In the present study, we have developed MFC panels for analysis of phenotype and activation status of mouse and human eosinophils in steady-state conditions or upon activation. In particular, we developed a panel for the characterization of mouse eosinophils ex vivo in tumor models and following stimulation with IL-33 in vitro, consisting of several monoclonal antibodies (MoAbs): Ly6G, Siglec-F, CD69, CD11b and CD44. In parallel, we designed a panel for human eosinophil activation, consisting of a commercial backbone dried antibody mixture, containing CD45, CD294, CD11b, CD15, CD16, CD33 and CD274 (PDL-1), CD62L and a lineage marker cocktail for lympho-monocyte exclusion, integrated with MoAbs detecting Siglec-8 along with the CD69 and CD63 activation markers, to stain whole blood samples stimulated in vitro. Moreover, a deep MFC of circulating granulocytes, including eosinophils, was performed on whole blood samples, in the context of a clinical study for the development of a therapeutic vaccine for breast cancer patients. Preliminary results indicated a statistically significant lower eosinophil rate in metastatic compared to primary breast cancer patients; lower levels of activated eosinophils (CD62L-) in both primary and metastatic patients, compared to age-matched, female healthy volunteers, as well as lack of different activated innate immune cells ($p = 0.001$ for CD62L-) in primary and metastatic patients versus healthy subjects. Although optimization and harmonisation will be crucial to guarantee reproducibility of data especially in multicentre trials, our MFC panels provide a starting tool for eosinophil characterization as immune biomarkers in pre-clinical models of disease and clinics.

ANALYSIS AND SORTING OF EXTRACELLULAR VESICLE SUBPOPULATIONS BY HIGH-RESOLUTION FLOW CYTOMETRY

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Exosomes are a subtype of small extracellular vesicles (sEV) formed within late endocytic compartments or multivesicular bodies (MVB) by inward budding of their limiting membrane into the lumen. Interest in exosomes has grown exponentially in recent years due to their crucial role in intercellular communication and to their potential clinical application in therapy and diagnosis. However, important aspects of the biology of exosomes remain unknown because of the wide heterogeneity among secreted vesicles. Thus, new reliable experimental strategies to identify specific exosome populations originating from MVB are needed. We developed a methodology to obtain fluorescent vesicles (Bodipy sEV) by using palmitic acid Bodipy FL C16 (C16) and lauric acid Bodipy 558-568 (C12), that emit fluorescence in the wavelength of green and red, respectively. When internalized by cells, they are converted into phospholipids and are incorporated into the bilayer of secreted vesicles that can be traced and counted through flow cytometry.
To characterize Bodipy sEV we first analyzed their protein and lipid profile and then sorted them to obtain a pure population of green fluorescent sEV. Sorted Bodipy sEV were analyzed by electron microscopy. We observed a homogeneous population of vesicles about 80 nm in size smaller than the bulk of secreted sEV expressing the exosomal markers CD63 and CD81 on their surface. Moreover, we successfully sorted green and red Bodipy sEV released by cells labeled with C16 and C12.
Finally, we have combined the C12 labelling technique with transfection using a DNA vector that expressed a mutant form of the HIV-1 Nef protein (NefG3C) fused with Green Fluorescent Protein (GFP). This particular variant of Nef contained a palmitoylation domain at its N-terminus, which served as a sEV-anchoring protein. Upon transfection and sorting, experimental data indicated that the cells released green and red sEVs along with double-labeled vesicles identified as exosomes. Taken together, these results demonstrate that the use of sorting is a valuable tool in obtaining pure population of sEV that can be further characterized and used to study their interaction with antigen presenting cells, like dendritic cells and macrophages.

FLOW CYTOMETRY CHARACTERIZATION OF TUMOR ASSOCIATED MACROPHAGES

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Tumor-associated macrophages (TAMs) play a central role in tumor growth, immune tolerance, and immunotherapy (IT) resistance. There is an urgent need for diagnostic tools for accurate qualitative, and quantitative assessment of these tumor-friendly cells. Immunophenotyping blood cells to evaluate the immune system is a powerful technology. Unfortunately, in depth knowledge of TAMs and a signature that can clearly and reliably identify them is still lacking. In the last decade, our lab has been involved in studying FKBP51 in immune oncology. FKBP51 promotes immune evasion through its short isoform, FKBP51s, a protein highly expressed in circulating monocytes of cancer patients resistant to IT. In this study we identified FKBP51s as univocal biomarker of TAMs. As a first approach, we performed ex vivo experiments of macrophage polarization with both the THP-1 macrophage cell line and primary monocytes to obtain classically activated M1-macrophages, alternatively polarized M2a macrophages, and the deactivated M2c-macrophages. The THP-1 immunophenotype revealed a strong CD45, HLA-DR, and CD86 expression associated with the M1 macrophages, while CD206, CD36, and Arginase 1 were highly expressed in M2 macrophages. Selective FKBP51s expression in M2c and M2a macrophages was observed by immunoblot and confirmed by flow cytometry. By immunophenotyping primary macrophages, we found Arginase 1 and the scavenger receptor CD36 significantly expressed in M2 macrophages, but some differences in M1 and M2 profiles were observed with respect to THP-1, thus highlighting the profound heterogeneity of primary samples. Particularly, the M2 markers CD163 and PD-L1 did not show significant differences in expression among the different profiles. Interestingly, we observed that the co-expression of CD163 or PD-L1 with FKBP51s appeared only in M2 macrophages, thus supporting the conclusion that FKBP5 splicing can make the difference in identifying this cell subset. To validate our in vitro results, we immunophenotyped TME and peripheral blood from 37 Glioblastoma patients, using classical M1 and M2 macrophage markers in conjunction with FKBP51s and flow cytometry. Analysis of TME highlighted a strong positivity for FKBP51s in tumor infiltrating macrophages and in the tumor cells. Similarly, immunophenotyping of peripheral blood showed co-expression of FKBP51s with typical M2 markers (PDL-1, CD169, CD163, CD206, CD36, Arginase) in CD14-gated cells. In conclusion, in the complex scenario of macrophage polarization, where membrane protein expression is heterogeneous and common determinants mark either M1 and M2, this spliced protein appears to be an M2 hallmark useful for a qualitative/quantitative assessment of TAMs in cancer patients.

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AGE-MATCHED REFERENCE VALUES FOR CIRCULATING NATURAL KILLER T (NKT)-LIKE CELLS

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Background and Aim

Natural Killer T (NKT)-like cells are a heterogeneous group of T lymphocytes characterized by the expression of CD56 and other NK antigens. In particular, these cells are capable to produce cytolytic molecules typical of the innate immunity compartment, so their imbalance could lead to pathologic conditions. To the best of our knowledge, there is a scarcity of reference values of these cells, especially for the pediatric population, where normal ranges for different age groups are still lacking. Therefore, in this study we have defined reference values of NKT-like cells, for adults and for pediatric subjects subdivided in 5 age ranges, following a CE-IVD laboratory work-up.

Materials and Methods

Peripheral EDTA blood samples from outpatients referred to our laboratory for immunophenotyping were processed with a lyse-no-wash protocol using MultitestTM 6-color TBNK Kit. Moreover, the expression of CD4 and CD8 on NKT-like cell population was also evaluated. Flow cytometry acquisition and analysis were performed with BD FACSLyric (FACSuite and FACSuite Clinical softwares v1.5, BD).

Outpatients without alteration of any laboratory test and healthy blood donors were used to determine NKT-like age-matched reference values. A total of 490 subjects divided into six age ranges were finally enrolled: n. 44 (0-15 months), n. 72 (15-24 months), n. 85 (2-5 years), n. 65 (5-10 years), n. 66 (10-16 years), n. 117 (>16 years) and n. 41 blood donors.

Both reference values of absolute NKT-like cell count (μl) were expressed as median and 5th and 95th percentiles.

Results

Our reference values for NKT-like cells as cells/μl were: 14/μl (3-52) for 0-15 months; 18/μl (3-48) for 15-24 months; 26/μl (10-68) for 2-5 years; 46/μl (12-114) for 5-10 years; 53/μl (15-153) for 10-16 years; 97/μl (27-225) for >16 years. Considering the percentage on total lymphocytes our reference values were: 0.2% (0.03-1.0) for 0-15 months; 0.3% (0.07-1.0) for 15-24 months; 0.7% (0.2-1.9) for 2-5 years; 1.5% (0.4-4.6) for 5-10 years; 2.4% (0.6-7.2) for 10-16 years; 4.8% (1.3-11.2) for >16 years.

NKT-like cell number increases with the age both in percentage and absolute count. Interestingly, while NKT-like/CD8+ cells increased with age, NKT-like/CD4+ cells showed an opposite trend. Similarly, double-positive cells (NKT-like/CD4+CD8+) gradually decreased with age, while double negative cells (NKT-like/CD4-CD8-) increased up to 16 years and then decreased in adults.

Conclusions

This work is a first step over the definition of robust reference values for NKT-like cell subpopulations under the CE-IVD conditions. Providing NKT-like cell number and frequency in the diagnostic report appears helpful to better delineate the immune profile of patients and to establish NKT-like cells involvement in different disorders.

BOTH DOWN-REGULATION AND IN VIVO BLOCKADE OF MGLUR5 RECEPTORS REDUCE THE REACTIVE PHENOTYPE OF SPINAL-CORD ASTROCYTES AND IMPROVES THE DISEASE PROGRESSION IN A MOUSE MODEL OF AMYOTROPHIC LATERAL SCLEROSIS

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Amyotrophic lateral sclerosis (ALS) is a lethal neurodegenerative disease characterized by a selective death of motor neurons (MNs). ALS is a multifactorial and non-cell autonomous disease, where the non-neuronal cells are involved in MNs degeneration. Glutamate-mediated excitotoxicity has been proposed as a major cause that trigger MNs degeneration. Although the etiopathogenesis is not fully understood, several studies demonstrated that damage within MNs is sustained by the degeneration of spinal cord astrocytes. The metabotropic glutamate receptor mGluR5 likely play a role in ALS, since it is over-expressed and functionally altered in different experimental model of ALS. In this work we investigate the effects of mGluR5 down-regulation on the reactive phenotype of astrocytes in ALS. We used here spinal cord astrocyte cultures from adult mice expressing the mutant human SOD1 with a Gly93Ala substitution (SOD1G93A), mice expressing half dose of mGluR5 (mGluR5+/-) and mice carrying the SOD1G93A transgene and lacking one allele of the mGluR5 encoding genes. Experiments with the FURA2 dye showed a higher cytosolic calcium concentration in SOD1G93A than in WT mice. mGluR5 knocking-down significantly reduced the excessive [Ca2+]i. Confocal microscopy revealed that the astrogliosis markers GFAP, vimentin and S100β were more expressed in SOD1G93A respect to WT mice and decreased in SOD1G93AmGluR5+/- mice. The same was true for the expression of the autophagy activation marker LC3-II. mGluR5 knocking-down also reduces the presence of misfolded-SOD1 protein. Thus, a lower constitutive level of mGluR5 had a positive impact in SOD1G93A mouse astrocytes, suggesting that mGluR5 may be a pharmacological target for cell specific therapeutic approaches in ALS. Based on these results, we tested the effect of the pharmacological blockade of mGluR5 in SOD1G93A mice by CTEP [2-chloro-4-((2,5-dimethyl–1-(4- (trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine], a mGluR5 negative allosteric modulator (NAM), starting at the early symptomatic stage of the disease (90 days of life). This treatment increased survival probability and improved motor abilities of mice, showing that blockade of mGluR5 has a significant impact in-vivo on ALS clinical outcome. In conclusion, our results suggest that mGluR5 may represent a target for the use of CTEP, or other mGluR5 NAMs, as promising drugs for the therapy for ALS.

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Oncology

GUADECITABINE INCREASES RESPONSE TO COMBINED ANTI-CTLA-4 AND ANTI-PD-1 TREATMENT IN MOUSE MELANOMA IN VIVO BY CONTROLLING THE RESPONSES OF T-CELLS, MYELOID DERIVED SUPPRESSOR CELLS AND NK CELLS

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The combination of PD-1 and CTLA-4 blockade has determined an improved overall survival (OS) rate for malignant melanoma at 3 years of 58% as compared to ipilimumab alone. Immune checkpoint blockers (ICB) limit the tumor's immune escape yet only for approximately a third of all tumors and, in most cases, for a limited amount of time. Several approaches to overcome resistance to ICB are being investigated among which the addition of epigenetic drugs that are expected to act on both immune and tumor cells. Guadecitabine is a dinucleotide prodrug of a decitabine linked via phosphodiester bond to a guanosine that showed promising results in the phase-1clinical trial, NIBIT-M4 (NCT02608437). We used the syngeneic B16F10 murine melanoma model to study the effects of immune checkpoint blocking antibodies against CTLA-4 and PD-1 in combination, with and without the addition of Guadecitabine. We comprehensively characterized the tumor's and the host's responses under different treatments by flow cytometry, multiplex immunofluorescence and methylation analysis. Guadecitabine in combination with ICBs significantly reduced tumor growth compared to ICB and Guadecitabine treatment. The demethylating drug led to a general DNA-demethylation and transcriptional modification of gene expression. In particular, Guadecitabine greatly enhanced the efficacy of combined ICBs by increasing effector memory CD8+ T cells, and reducing tumor infiltrating regulatory T cells and MDSC cells, in the TME. These results indicate Guadecitabine as a promising epigenetic drug to be added to ICB therapy.

ANALYSIS OF EXTRACELLULAR VESICLE CARGO SECRETED BY GLIOBLASTOMA CANCER CELL MODELS IN VITRO AND IN VIVO

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Small extracellular vesicles (sEVs) mediate intercellular communication in both normal and tumor cells. sEVs derived from glioblastoma (GBM), one of the most aggressive and lethal forms of primary brain tumors, release molecules that alter the surrounding environment to favor tumor invasion. Important molecular effectors of this tumor promoting activity are microRNAs (miRNAs) and proteins.

We studied the miRNA (by Agilent microarray technology) and protein (by mass spectrometry) content of sEVs derived from three GBM cancer stem cell (CSC) lines originated from primary human tumors, in comparison with two established GBM cell lines (U87 and U373). The isolated sEVs were counted and characterized by FACS, for the presence of surface EV specific markers (e.g. CD81, CD9). We found a total of 854 miRNAs contained in the sEVs of our samples, of which 195 (23%) were in common among the three cell types. The analysis of such miRNAs indicate that U87 and U373 cell line-sEVs are selectively enriched for miRNAs that are known to display tumor suppressor activity. While, their protein cargo is enriched with oncoproteins and tumor-associated proteins. Conversely, among the most up-regulated miRNAs in CSC-sEVs, we also found pro-tumor miRNAs and proteins related to stemness, cell proliferation, and apoptosis. These data show that the miRNA and protein contents of sEVs are not functionally correlated, being miRNAs linked to pathways that control transcription and presumably regulate gene expression in recipient cells. In contrast, proteins present in EVs appear to be involved in a broad spectrum of cellular signal transduction pathways that may control how recipient cells respond to external signals.

The characterization of EVs secreted by tumor cells, to identify molecular signatures for tumor diagnosis has a great potential in the liquid biopsy landscape, which could possibly replace the more costly and invasive tissue biopsies in the near future. Recently, we developed a human GBM xenograft mouse model, using the U87 MG cell line, to accurately identify the sEVs specifically secreted in the bloodstream by the tumor cells from the brain. We traced the secreted EVs by transfecting the U87MG cells with a construct coding for a red fluorescent (dTomato, dT) CD81 fusion protein. CD81 is a tetraspanin specifically expressed on the sEV surface. After transfection, to enrich the dT-CD81 cell population, we sorted the fluorescent cells and expanded them in vitro. We then characterized the sEVs released by the U87 MG-dT-CD81 cell clone by microscopy, WB and FACS after immunocapturing the vesicles with magnetic beads. Then, we injected the U87MG-dT-CD81 cells in NSG mouse brains and purified circulating fluorescent sEVs. FACS analysis revealed the presence of about 1% of human fluorescent sEVs in the mouse plasma and we sorted them to profile miRNAs by RNASeq.

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DNA-PK INHIBITION SUSTAINS IMMUNOTHERAPY RESPONSE INDUCING NON-CANONICAL ACTIVATION OF STING PATHWAY THROUGH MAVS SIGNALING IN SCLC

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Introduction

Small Cell Lung Cancer (SCLC) is a hard-to-treat cancer type due to high aggressiveness and biological features. Several studies demonstrated that the addition of DNA damaging therapies (chemo-, radiotherapy or DNA Damage Repair Inhibitors, DDRi) to immunotherapy (IO) increases antitumor immune response via activation of Stimulator of Interferon Genes (STING), the major innate immune pathway. As a result, STING agonists are proving to be highly active immune-adjuvant agents in the treatment of both infectious diseases and cancer. Given the heterogeneity of SCLC and the possibility of switching between subtypes under treatment pressure, we hypothesize that DDRi may induce simultaneous activation of multiple innate immune pathways and sensitize otherwise immune-resistant SCLC. Here, we show that human PBMC-derived lymphocytes mount innate immune responses after DDRi involving a non-canonical STING activation mediated by DNA-PK inhibition, leading to assembly of an alternative STING signalling complex including mitochondrial antiviral signalling protein (MAVS).

Material and method

PBMCs from SCLC patients (pre- and post-chemoimmunotherapy, CIT) or healthy donors were isolated and grown for 5 days alone or in coculture with H446 SCLC cell line spheroids. After 48h, DNA-PK-I and/or ENPP1-I (2 μM) plus atezolizumab (10 μg/ml) were added for further 72h. STING, MAVS and PDL1 expression was assessed at mRNA and protein levels in SCLC and PBMC. Indirect immunofluorescence (IF) and co-immunoprecipitation (IP) experiments were used to assess the subcellular localisation and physical interaction of STING and MAVS in immune cells. After coculture, spheroid diameter was assessed and spheroid-infiltrating (IN) and medium-remaining (OUT) immune cells were mechanically separated and analysed by flow cytometry (FC).

Results and discussion

The expression of STING, MAVS and PDL1 was significantly increased ($p<0.0005$) in DNA-PK-I treated SCLC PBMCs compared to untreated cells. Higher levels of STING/MAVS were found in PBMCs collected from patients after CIT. Increased mitochondrial recruitment of STING was found in DNA-PK-I treated immune cells. IP analysis showed a physical interaction between MAVS and STING in DNA-PK-I PBMC-derived lymphocytes from SCLC patients. After ENPP1-I treatment, no STING translocation or interaction with MAVS was detected. 3D coculture was used to verify the efficacy of the DDR pathway in enhancing STING-MAVS-mediated antitumour immunity. The strategy using DNA-PK-I plus IO strongly increased the spheroid infiltration of lymphocytes (CD3+CD45+) when comparing the cellular proportions IN and OUT to control and highly reduced spheroids diameter after 5 days, suggesting that immune cells infiltration disrupted the tumour structure.

Conclusion

We provide evidence that inhibiting DNA-PK recruits STING to mitochondria to interact with MAVS and mediate a direct antitumour effect. Our results suggest that DNA-PK inhibitors may be a therapeutic strategy to improve the response to IO in SCLC patients.

IDENTIFICATION OF HERV-K EXPRESSION IN PATHOLOGICAL LYMPHOCYTES SUBPOPULATION OF CHRONIC LYMPHOCYTIC LEUKAEMIA

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Background: The dysregulated expression of human endogenous retrovirus K (HERV-K) at transcriptional and protein levels, as well as viral particle production, have been found in tissues, sera, and cell lines isolated from different types of tumors. The mechanisms underlying HERV-K oncogenic activity could depend on the expression of oncogenic viral proteins, on the induced immune escape mechanisms, on the regulation of gene expression mediated by the long terminal repeat sequences or by the ability of retro-transposition determining genomic instability and alteration of the expression of neighboring genes. In the field of oncohematology some studies have identified alterations of HERVs messengers and proteins expression in human lymphoid leukemic cells and the presence of circulating antibodies to HERV-K. On these bases, the objective of the study was to evaluate the potential use of distinct HERVs families as biomarkers of disease and prognosis of chronic lymphocytic leukemia (CLL). **Materials and methods:** Peripheral blood mononuclear cells were isolated from peripheral venous blood of 75 patients with CLL and 49 healthy donors (HDs). CLL patients were divided according to the treatment regimen they received. The expression of the ENV gene of HERV-K (HML-2), HERV-H, HERV-W, pathogenic HERV-W and HEMO were analyzed by Real Time PCR. In addition, the percentage of HERV-K ENV-positive cells in B lymphocyte subpopulations (CD19+CD5+) and in association with the negative prognostic marker CD38 was characterized by flow cytometry analysis. **Results:** The molecular analysis showed significantly higher expression of HERVs in patients compared with HDs. Significant differences in all genes analyzed between untreated patients and those treated with chemotherapeutic or biological drugs patients were found. Moreover, a higher level of ENV HERV-K protein in CD19+CD5+ cells of CLL patients and its modulation in the presence of therapy were found. Association between transcriptional and protein expression of HERV-K ENV and unfavorable prognostic factors were found. **Discussion and conclusion:** These results could help to clarify the CLL complexity, proposing HERVs and as potential new diagnostic and prognostic markers, suggesting their involvement in the etiopathogenesis of the disease. This scenario opens new avenues for investigating the use of HERVs as potential therapeutics in combination with standard therapies

EVALUATING THE ADIPORON EFFICACY IN SENSITIVE AND GEMCITABINE RESISTANT PANCREATIC DUCTAL ADENOCARCINOMA MIAPACA-2 CELLS

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Introduction: Pancreatic ductal adenocarcinoma (PDAC) is the most common malignant disorder affecting pancreas, and leads to nearly half a million deaths per year worldwide. Besides radiation and surgery, Gemcitabine (Gem) represents the conventional antineoplastic drug used to treat PDAC. Unfortunately, the limited success rate of Gem treatment and the relative ease in developing chemoresistance warrant for more effective therapeutic approaches in PDAC. The first synthetic orally active adiponectin receptor agonist AdipoRon (AdipoR) has shown to possess cardioprotective, anti-diabetic, and anticancer activities. Here, we explore the antiproliferative impact of AdipoR, as well as the chance of increasing sensitivity, in both MIA PaCa-2 and MIA PaCa-2 Gem resistant (MIA PaCa-2 Gem-Res) human PDAC cells.
Materials and Methods: PDAC cells were exposed to AdipoR (10 μg/ml) alone or in combination with Gem (15 nM) for up to 48 hours. The outcome were evaluated by measuring cell growth and colony forming ability using growth curves and chlorogenic assays, respectively. Fluorescence activated cell sorting (FACS) analysis was performed with the purpose of defining the drug-induced effects on cell phases’ distribution. MIA PaCa-2 Gem-Res were obtained exposing MIA PaCa-2 cells to increasing Gem concentrations. FACS analysis was employed to confirm Gem resistance, as well as to investigate the AdipoR consequences in these cells.
Results: AdipoR improved Gem response in both MIA PaCa-2 and MIA PaCa-2 Gem-Res cells, with no additional cytotoxic effects. FACS analysis of MIA PaCa-2 cells showed intermediate features in combination treatment (Combo) with respect to single agents. Specifically, Combo displayed a G0/G1 increase closer to AdipoR and a S-phase accumulation similar to Gem. In MIA PaCa-2 Gem-Res instead, AdipoR persisted in braking cell cycle progression, while no differences were observed between Gem and the untreated counterpart. But even more interesting, reducing both S and G2/M phases, the concomitant administration of AdipoR and Gem further enhanced the G0/G1 accumulation compared with AdipoR alone in MIA PaCa-2 Gem-Res cells. In addition, AdipoR reduced clonogenic potential by 45 and 55% in response to AdipoR and Combo, respectively.
Conclusion: Our results recognize additional and newly AdipoR therapeutic usages in PDAC, either as a potential partner in Gem-based therapy or as subsequent line of treatment in Gem resistance cells.

CIRCULATING CD137+ T CELL LEVELS ARE CORRELATED WITH RESPONSE TO PEMBROLIZUMAB TREATMENT IN ADVANCED HEAD AND NECK CANCER PATIENTS

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Abstract: Pembrolizumab, an anti-PD-1 antibody, has been approved as first-line treatment for recurrent or metastatic head and neck squamous cell carcinoma ((R/M) HNSCC). However, only a minority of patients benefit from immunotherapy, which highlight the need to identify novel biomarkers to optimize treatment strategies. CD137+T cells have been identified as tumour-specific T cells correlated with immunotherapy responses in several solid tumours. In this study, we investigated the role of circulating CD137+ T cells in (R/M) HNSCC patients undergoing pembrolizumab treatment.
PBMCs obtained from 40 (R/M) HNSCC patients with a PD-L1 combined-positive-score (CPS)>1 were analysed at baseline via cytofluorimetry for the expression of CD137, and it was found that the percentage of CD3+CD137+ cells is correlated with the clinical benefit rate (CBR), PFS, and OS.
The results show that levels of circulating CD137+ T cells are significantly higher in responder patients than in non-responders (p=0.03). Moreover, patients with CD3+CD137+ percentage >1.65% had prolonged OS (p=0.02) and PFS (p=0.02). Multivariate analysis, on a combination of biological and clinical parameters, showed that high levels of CD3+CD137+ cells (>1.65%) and performance status (PS)=0 are independent prognostic factors of PFS (CD137+T cells, p=0.007; PS, p=0.002) and OS (CD137+T cells, p=0.006; PS, p=0.001).
Our results suggest that levels of circulating CD137+ T cells could serve as biomarkers for predicting the response of (R/M) HNSCC patients to pembrolizumab treatment, thus contributing to the success of anti-cancer treatment.

Environmental Sciences and Biotechnology

EPIGENETICS OF THERMAL STRESS: A FLOW CYTOMETRY AND ELECTRON MICROSCOPY APPROACH

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NIntroduction

The cellular environment of living organisms constantly encounters several agents which have the potential to perturbate the cellular homeostasis, causing damage at multiple levels. Stress response and adaptation is based on profound modifications of the gene expression pattern, which is achieved acting at different levels, such as structural and architectural nuclear rearrangement and epigenetic modifications. The crosstalk between genome-epigenome, its role in shaping the epigenetic landscape and the stress-mediated cellular response has not yet been fully elucidated. In this complex framework, an important starting point is to determine the impact of frequently encountered stresses on the histone epigenetic layer, how the latter varies according to each perturbation, consequently changing the chromatin organization. Whereas various stress responses have largely been investigated, major features of certain insults remain obscure and elusive. For example, temperature alterations are among the most common stresses cell must deal with. Hyperthermia is widely studied as the response is similarly activated by a range of cellular stressors and by pathophysiological conditions. On the contrary, hypothermia has been far less studied, but it is currently gaining major relevance for its clinical, therapeutical, and industrial outcomes.

Aim

This work aims at investigating the relationship between thermal stress and cell structural and epigenetic response by focusing our attention on histone post-translational modifications, on their influence on chromatin organization following stress exposure as well as on the level of cell damage triggered by the thermal stress.

Methods

Flow cytometry assay of HeLa cells stained with propidium iodide was performed to evaluate the impact of the thermal shocks on cell viability. Electron microscopy morphological analysis and immunogold labelling were adopted to investigate alterations at the ultrastructural level.

Results

The combination of flow cytometry and electron microscopy highlights the distinct structural and epigenetic fingerprints in response to the two types of insult. Hyperthermia is strictly associated with increments in histone methylation and induces a more compact chromatin organization. Conversely, hypothermia does not determine a univocal epigenetic response: indeed, different epigenetic markers are contemporarily exhibited, confirming the delicate gene expression regulation pathways required when dealing with severe condition alterations.

Conclusions

Cells react differently to stresses in terms of histone markers distribution, chromatin organization and damage response. Given the involvement of epigenetic and chromatin organization in stress adaptation, elucidating the role of histone-mediated gene expression regulation in the transcription of genes involved in protective or pathogenic pathways may underline potential outcomes for medical, pathological, and commercial applications.

MICROBIAL COMMUNITY PROFILES AND ANTIBIOTIC RESISTANCE POTENTIAL IN A MARINE AQUACULTURE SYSTEM

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Aquaculture represents a key source of fish food for human consumption, but the environmental impact of fish farming practices is still poorly investigated. One of the main concerns is the increased nutrient and pollution levels of surrounding waters and coastal areas, including local eutrophication conditions and the dispersal of chemicals and antibiotics used to prevent fish diseases. In the frame of the project ARENA (https://www.jpi-arena.eu/it/), we aim at producing an overview of microbial community profiles and antibiotic resistance potential in a marine aquaculture setting located in a highly anthropized coastal environment with multiple pollution sources (e.g., urban settings). Flow cytometry was used to identify and count the planktonic, benthic, and fish-associated microorganisms. The Biolog assays (Ecoplates, MT2, 11C and 12B 96-wells microplates) were used to explore the community level physiological profiles and the occurrence of potential microbial resistance to 16 major classes of antibiotics (i.e., Aminoglycosides, Glycopeptides, Tetracyclines, Lincosamides, β-Lactams, Quinolones, Polymyxines, Phenicols, Sulfonamides, Macrolides, Aminocoumarines, Pteridines, Ammonium salts, Aminocyclitol, Hydroxammases, Rifamicines). Our results indicated a relatively high and widespread microbial load level in all environmental samples (waters, sediments), along with potentially expressed antibiotic resistance. At the end of the tourist season, sediments affected by urban runoff showed the highest metabolic functional diversity and the largest resistance expression toward antibiotics. The study findings are intended to provide knowledge and tools for future operative efforts to decrease the spread and magnification of antibiotic resistant bacteria and microbial contamination in aquaculture.

FLUORESCENCE-GUIDED DETECTION OF MICRO- AND NANO-PLASTICS IN ENVIRONMENTAL SAMPLES

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Plastic pollution represents a global environmental issue with relevant associated risks for ecosystem and human health. Fluorescence-based detection techniques have demonstrated to be a rapid and reliable tracking method for micro and nanoplastics (MNPs) identification in numerous studies by combining pre-treatment procedures, specific staining protocols, and advanced analytical instruments (including flow cytometry). However, despite an increasing scientific attention, the detection of plastic debris in the submicron and nano size range is still challenging when targeting environmental complex samples. In particular, the suitable approaches are not consistently harmonized and generally case/system-specific, with detrimental consequences on data reproducibility, accuracy, and comparability.
In this work, we aim to provide background information on the existing fluorescence-based approaches to assess the occurrence of MNPs in environmental samples of different origin, including potentialities offers by flow cytometry to detect small plastic particles as demonstrated by our recent study. Overall, fluorescence-based techniques are likely offering promising results and unique analytical opportunities for the detection and quantification of environmental MNPs even at the nano-size domain.

CANCER INDUCES VERY HIGH LEVELS OF SPERM OXIDATIVE STRESS

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It has been extensively shown that cancer impairs spermatogenesis and several studies observed also an increase of sperm DNA fragmentation (sDF), albeit not confirmed by others. To our best knowledge, so far no investigation has been conducted on sperm oxidative stress. In this study we aimed to detect both sDF and oxidative stress levels in spermatozoa of patients affected by testicular and haematological cancer. For oxidative stress we used a novel flow cytometric method detecting both viable (ROS production in viable sperm fraction/viable spermatozoa) and total (ROS production in viable sperm fraction/total spermatozoa) oxidative stress. We found that cancer (22.50[17.00-26.75]%, n=85) increased sDF respect to the two control groups: normozoospermic subfertile subjects (12.75[8.63-14.88]%, n=52, p<.001) and healthy donors (8.50[7.00-14.00]%, n=19, p<.001). The induction of viable oxidative stress (n=96) with cancer was even higher: 36.60[24.05-58.65]% versus 11.10[8.63-14.90]% in normozoospermic subfertile subjects (p<.001) and 9.60[8.00-14.03]% in healthy donors (p<.001). Similar, albeit lower, differences were found for total oxidative stress: 23.90[14.27-37.63]% in cancer vs 8.15[6.23-11.08]% in normozoospermic subfertile subjects (p<.001) and 6.00[7.46-10.29]% in healthy donors (p<.001). When we correlated the amounts of sDF to those of oxidative stress we observed a sharp association between the two parameters when we considered all subjects (cancer patients and controls) (r=0.591, p<.001, n=134), but we failed to reveal a correlation when only cancer patients were studied (r=0.200; p>.05, n=63). When we compared the main subtypes of testicular cancer, we observed a clear, albeit not significant (p=0.077), trend to higher viable oxidative stress in non-seminoma vs seminoma cancer, whereas no difference in sDF levels was revealed. Similarly, no difference in either oxidative stress and sDF amount was observed in patients with testicular cancer before and after orchiectomy.
In conclusion, cancer highly increases sperm oxidative stress, beside to sDF levels. It is possible that alternative mechanisms to oxidative attack are responsible for increase of sDF by cancer. Since the levels of sperm oxidative stress might affect the outcomes of sperm cryopreservation, cancer treatments and even sperm epigenoma, future studies will tell us whether detection of oxidative could be of help in managing the reproductive issues of cancer patients.

PLANKTONIC MICROBIAL COMMUNITY PATTERNS IN WATERS OF AN EAST AFRICAN SODA LAKE (SONACHI, KENYA)

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Soda lakes are highly alkaline (pH 9-11) and characterized by high concentrations of dissolved sodium carbonate. These extreme conditions create a unique environment for planktonic microbial communities that are adapted to survive in extreme conditions. These extremophilic microorganisms play important roles in the carbon and nutrient cycling in lake waters and are important models for studying the limits of life on Earth.
Here, we present the microbial community patterns found along the water column of the soda lake Sonachi in the East African Rift Valley (Kenya). As assessed by flow cytometry, microscopy, and molecular methods, numerous suspended aggregates promoted the interactions between Bacteria, Cyanobacteria, and Archaea. Moreover, the formation and origin of microbial aggregates appear to be critical in connecting the lake compartments through biomass and organic matter transfer. Our findings provide insights into understanding how hydrogeochemical features and microbial community profiles of a meromictic soda lake could promote methane emission.

CULTURABILITY VS VIABILITY: ADVANCED MONITORING OF THE ANTIMICROBIAL AND ANTI-BIOFILM ACTIVITY OF NATURAL COMPOUNDS TO BE INCORPORATED IN ACTIVE PACKAGING

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Background: The increasing focus on packaged and ready-to-eat products has enhanced the risks associated with foodborne illness, demanding the development of advanced microbiological monitoring systems. Natural bioactive compounds incorporated in innovative packaging represent an extremely promising alternative for preventing food microbial contamination and spoilage and for extending products' shelf-life.

Objectives: The aim of this study was to assess the antimicrobial and antibiofilm activity of selected bioactive compounds against foodborne pathogens and spoilage microorganisms. Both traditional culture-based method and Flow Cytometry (FCM) were applied to evaluate the potentiated action of Lactobacillus plantarum probiotic strain together with natural antimicrobial molecules on the culturability and viability of pathogenic and spoilage strains.

Methods: Traditional microbiological methods and FCM [1] were applied to evaluate the effect of antimicrobial treatment of natural compounds (Thyme essential oil (EO), Origanum EO, Basil EO, Citrus Limon EO, Carvacrol, Limonene, Gallium(III) nitrate hydrate, Nisin) at different concentration (50-100-250-500 ppm) against the planktonic and sessile cells of four selected strains (Escherichia coli ATCC25922, Pseudomonas fluorescens ATCC 13525, Listeria monocytogenes 54ly, Lactobacillus Plantarum DSM 20174).

Results: The results obtained showed a higher efficacy of Carvacrol and Thyme EO, highlighting an overestimation of the dead population using the culture-based method; in fact, when the FCM method was applied, the prevalence of injured bacterial cells in a viable but non-culturable (VBNC) state was observed. When bioactive molecules were applied to a preformed biofilm of L. plantarum, an enhanced effect was observed. The latter could represent a promising alternative to functionalize antimicrobial ready-to-eat products packaging.

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New Technologies and Cytometric Methodologies

CHARACTERIZATION OF BIOMIMETIC NANOPARTICLES AND ANALYSES OF THEIR HOMOTYPIC TUMORAL TARGETING CAPABILITY BY FLOW CYTOMETRY

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Nanotechnology approaches offer the opportunity to specifically target cancer cells for delivering active therapeutic agents, with important applications in the growing field of immunotherapies.
In our research, biomimetic nanovectors are produced, by using polymeric NPs based on carboxyl-terminated polycaprolactone (PCL) that are covered by cancer cell membrane, thus conferring to the assembled NP the ability to entirely replicate the surface antigenic diversity of source cells. Biomimetic NPs are physically characterized by DLS and TEM analysis, as well as through a cytofluorimeter CytoFLEX S (Beckman-Coulter) equipped with a violet laser detector to collect side scatter (VSSC). By flow cytometry measurements on biomimetic NPs important parameters such as number and size distribution of different nanoformulations are extracted, including PCL-core NPs, membrane vesicles and assembled biomimetic NPs by employing a template with gates for different sizes. The correct coating of PCL NPs with cancer cell membranes is confirmed by DLS analyses thanks to changes in both size and surface charge, and morphologically by TEM images for the presence of a layer surrounding NPs core. The purity and retention of cancer cell membrane proteins on biomimetic NPs is assessed through SDS-PAGE and Western blotting. Also, anticancer immune responses and specific targeting capabilities through homotypic binding are deeply investigated. In particular, cellular uptake of biomimetic NPs over time, and homotypic binding with different cell lines are assessed by flow cytometric analyses and Confocal Laser Scanning Microscopy imaging. These investigations confirm the higher internalization rates of biomimetic-NPs in their source cells, when compared to other cell lines, thus confirming the self-recognition capability typical of cancer. In conclusion, resulting biomimetic NPs can be used to deliver tumour-associated antigens to APC cells or to homotypically target cancer cells for drug/ gene delivery thanks to a cancer-targeting strategy based on the intrinsic self-adhesive properties of cancer cell membranes. By coating the nanocore with cancer cell membranes, it is possible to take advantage of this cell-to-cell adhesion for cancer cells targeting in precision medicine.
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RAPID EVALUATION OF T CELL CLONALITY IN THE DIAGNOSTIC WORK-UP OF MATURE T CELL NEOPLASMS: TRBC1-BASED FLOW CYTOMETRIC ASSAY EXPERIENCE

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The identification of mature T cell neoplasms by flow cytometry is often challenging, due to overlapping features with reactive T cells and limitations of currently available T cell clonality assays. The description of an antibody specific for one of two mutually exclusive T cell receptor (TCR) β-chain constant regions (TRBC1) provides an opportunity to facilitate the detection of clonal TCRαβ+ T cells based on TRBC-restriction. Here we prospectively analyzed 14 healthy controls and 63 patients with the flow cytometry protocol currently used for suspected T cell neoplasm implemented with immunostaining targeting TRBC1. Specimens were firstly classified in 3 groups based on clinical records data, laboratory findings and immunophenotypic features. T cell clonality was assessed by TCR Vβ repertoire analysis and the new rapid TRBC1 assay. Results showed that TRBC1 unimodal expression was unequivocally associated with samples presenting with immunophenotypic aberrancies. Moreover, we demonstrated that the use of TRBC1 is useful in solving uncertain cases and confirmed the high sensitivity of the method in identifying small T cell clones of uncertain significance (T-CUS). Finally, we found a high degree of concordance (97%) comparing the currently available clonality assessment methods with the proposed new method. In conclusion, our results provided real-life evidence of the utility of TRBC1 introduction in the flow cytometric clonality evaluation for the routine diagnostic work-up of T cell neoplasms.

SAMPLE PREPARATION AFFECTS FLUORESCENCE SIGNAL IN MULTICOLOR FLOW CYTOMETRY

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Flow cytometry is an evolving technology largely used in basic research as well as in translational research and clinical practice. The development of new fluorophores together with the implementation of instruments have contributed to the growth of the so-called next generation flow. Moreover, the improving complexity of the antibody panels for immunophenotyping runs in parallel to the increase of the experiment set up thus leading the appropriate antibody and fluorochrome selection combined with optimized staining protocols, as imperative steps for a successful in-depth flow cytometry analysis.
Here, we analyze some factors that might affect the fluorescence signal from a wide range of newborn class of fluorochromes. In particular, we focused on two critical aspects: sample washing and fixation.
First, we observed a reduction of the background signal upon increasing the number of sample washes, probably due to the reduction of the antibody aspecific binding.
As second topic, we analyzed the impact of aldehyde-based fixatives on both cell morphology and fluorescence signal thus defining PFA 1% for two hours at room temperature as the best condition to preserve cell shape and size and to minimize the formation of fluorescent formaldehyde adducts.
Finally, in the effort to highlight whether alcohol-based fixatives, mainly used for phosphoprotein analysis, affect the fluorescence signals, we tested their impact over time on 56 different fluorescent dyes. Interestingly, we observed a dramatic bleaching of some dyes thus limiting their use in multicolor panels that require these kind of fixatives.
Altogether, these data demonstrate the key value of the sample preparation for a good multicolor flow cytometry set up, thereby minimizing misleading data.

HIGH-THROUGHPUT BLOOD ANALYSIS USING SPIRAL MICROFLUIDICS TESTED BY IMAGING FLOW CYTOMETRY AMNIS

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A detailed procedure for blood analysis using spiral microfluidic devices is presented that allows size-based isolation of blood cell populations using hydrodynamic forces present in curvilinear microchannels. Spirals are used to separate cells according to their size due to the so-called Dean forces. Channel dimension, number of spirals and diameter of the curvature influence the sorting effect. The sample is introduced through a central inlet and fractions with particles of different size can be received at the different outlet ports. The chip contains four sorting structures with different features such as: number of turns, number of outlets, channel width and channel depth. Here, we test blood sample from healthy donors with a specific chip structure with 12 number of turns, 6 number of outlets, channel width of 80 µm and a channel depth of 50 µm. In order to assess the performance of the procedure for separating blood cell populations, the innovative imaging flow cytometry Amnis is employed. The FlowSight system (Luminex) is an advanced imaging flow cytometer, combining features of fluorescent microscopy and flow cytometry. The instrument is accompanied by a dedicated image analysis software (IDEAS), which allows advanced quantification of intensity, location, morphology, population statistics, and more, within tens of thousands of cells per sample. This allows feature finder tools for the identification of parameters that allow us to discriminate between different cell subpopulations based on the selection of few representative events. This instrument gives rise to novel applications that were difficult to achieve by either conventional flow cytometry or microscopy.
Here, the results of the procedure for sorting blood components are presented and discussed. The fast-processing time and the ability to collect and sort blood populations and to detect possible pathological cells from a small patient blood volume would open in future to a broad range of biomedical applications, for example in aerospace field. Indeed, there is a great demand of new technologies to monitor the human health during space missions avoiding complex sample preparations and suppling reliable results without highly skilled personnel. Space environment introduces several different hazards (distance, confinement, hostile/closed environments, radiation, and microgravity) that have health risks and consequences that span multiple organ systems. The astronauts are subjected to psycho-physical stress and possible inflammatory states. The knowledge gained in the human spaceflight field has highlighted the importance of frequent monitoring of astronaut's health. Early diagnoses and in situ medical interventions could mitigate the risk of health consequences induced by space exposure. In the context of long-term human missions, our revolutionary approach could be extremely useful to monitor the astronaut health after space missions and patient health on the Earth.

SINGLE-CELL ANALYSES AND IMMUNOLOGICAL PATTERNS IN IBD PATIENTS

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Inflammatory bowel disease (IBD) is an umbrella term that covers several clinical conditions, with two of them being the most common: Ulcerative Colitis (UC), which is limited to the colonic mucosa; and Crohn's Disease (CD), which potentially affects any segment of the gastrointestinal tract from the mouth to the anus.
Both UC and CD are chronic, lifelong inflammatory diseases with a pattern of flare-ups and remission. While we have learned a lot about the predisposing factors, clinical symptoms, pathways, pathology, and genetics, the exact cause of these diseases remains unknown. The number of clinical trials aimed at the discovery of novel therapeutic options is increasing annually. But with more trials comes increasing complexity and challenges.
Increased expression of CD39 by peripheral blood Treg is observed in the setting of clinical and endoscopic remission in inflammatory bowel disease. Deficiency of CD39 expression by T reg can be linked to inability to suppress experimental colitis. CD39 expression endows Th17 cells with immunosuppressive properties and promotes the transition to a less pathogenic phenotype. (1)
As a CD4+ T cell subset, Th17 cells play dual roles in the pathogenesis of IBD (mainly a proinflammatory role). Th17 cells can not only protect the intestinal mucosa by keeping the balance of the immune microenvironment but also exacerbate the intestinal inflammatory response through proinflammatory cytokines. The differentiation process of Th17 cells can be divided into three stages: IL-6 and TGF-β initiate the differentiation of Th17 cells, IL-21 expands the differentiation state of Th17 cells, and IL-23 maintains the stable maturation of Th17 cells during the later stage of differentiation.
A previously unrecognised imbalance exists between cellular and humoral immunity to the microbiota in IBD, with loss of mucosal T cell-mediated barrier immunity and uncontrolled antibody responses. Regulatory function of Trm may explain their association with intestinal health. Promoting Trm and their interaction with dendritic cells, rather than immunosuppression, may reinforce tissue immunity, improve barrier function, and prevent B cell dysfunction in microbiota-associated disease and IBD aetiology.
We recently start a protocol aiming to better describe functional and inflammatory patterns in ulcerative colitis patient. We, in fact start to evaluate along typical lymphocyte subpopultation CD45RA antibody to facilitate further characterization or sorting of naïve (CD45RA+) or activated/memory (CD45RA-) CD4+Foxp3+ or CD4+Foxp3- T lymphocytes. On the other hand, we start to emphasize naïve and memory T cells, the panel comprises the following fluorochrome-conjugated antibodies: CD197, CD95, CD27, CD3, and CD8.
A primary hypothesis consist to look for residential and mucosal CD8 lymphocyte before and after biological treatment in order to correlate their frequency with inflammation and consequential mucosa healing. (Figure 1)

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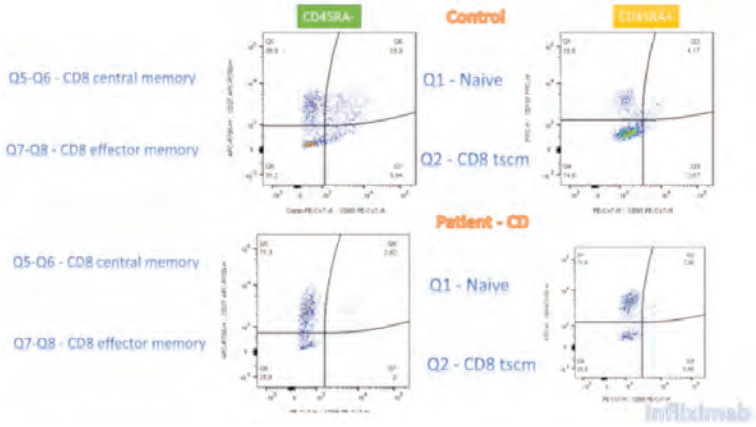
Figure 1. CD8 central and effector memory in patient with IBD



A NEW RNA-BASED FLOW CYTOMETRIC METHOD TO ASSESS POST-TRANSPLANT CHIMERISM IN PEDIATRIC PATIENTS UNDERGOING ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Background: Mixed chimerism is a condition of coexisting donor and recipient hematopoietic cells after hematopoietic stem cell transplantation (HSCT). Since it is of pivotal importance for clinical management of patients, chimerism analyses are routinely performed on total peripheral blood or bone marrow cells, but also on sorted cell subsets as this can be predictive of graft rejection.
Aim: We propose a novel flow-cytometric based method to assess chimerism in patients with donor-recipient sex-mismatch.
Material and methods: PrimeFlow RNA assay allows to detect the presence of a mRNA through flow cytometry. We identified a transcript derived from a Y chromosome gene as target, KDM5D, allowing to distinguish male and female cells. The signal produced by the mRNA was acquired through Aurora flow cytometer, 5 Laser (Cytek®). The same samples were in parallel analyzed by short tandem repeats PCR (STR-PCR) which is the gold standard method for chimerism analysis.
Preliminary data: Following an initial set-up phase, we performed flow-cytometric chimerism analysis on HSCT patients' samples (n=8) and obtained comparable results to gold standard method STR-PCR on total peripheral blood cells and in lymphoid and myeloid fractions. Subsequently, we exploited the potential of the technique by developing ad hoc cytofluorimetric panels to assess chimerism in T and B lymphocyte subsets. Compared to the gold standard, our method produces more information about the main immune cell subsets without the need of sorting the cells.
In conclusion, custom cytofluorimetric panel could be designed for the immune cell subset of interest. This paves the way for in-depth studies of post-HSCT immune reconstitution.

Cell Cycle and Apoptosis

THE POTENTIAL ROLE OF POMEGRANATE EXTRACT OBTAINED BY HYDRODYNAMIC CAVITATION AS ADJUVANT IN CANCER THERAPIES

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Introduction Natural bioactive compounds possess anti-cancer properties including the ability to kill cancer cells without being toxic to normal cells. Most fruit is rich of polyphenols as flavonoids and tannins, which interfere with carcinogenicity. In this study, the possible anticancer capacity of an extract of whole pomegranate obtained by hydrodynamic cavitation (HC) was evaluated. It's known that pomegranate contains high amount of polyphenols such as punicalagin, an ellagitannin with antioxidant activity and influence on modulation of multiple signaling pathways, suggesting its use in combination with standard therapies. HC involves the formation, growth and collapse of vapor-filled microbubbles in a circulating liquid-solid mixture subjected to an oscillating pressure field, leading to the localized release of energy in extreme density and in the mechanical, thermal and chemical forms. HC is applied in water remediation, disinfection or extraction of natural products and is used as microbiological stabilisation technique. Materials and methods After different times of HC, phenolic content of five pomegranate extracts (from MGM1 to MGM5) were assessed by Folin-Ciocalteau assay. A breast cancer cell line (AU565-PAR) was treated with MGM3 at different concentration for 48 h and then cellular growth was evaluated by trypan blue exclusion test. Cell death by apoptosis was assessed by propidium iodide while the early apoptosis by annexin V staining by flow cytometry. The ROS production was analysed using DCFDA fluorogenic probe by flow cytometry. Furthermore, growth and cell death were examined in healthy donors lymphomonocytes (PBMCs) after MGM3 treatment to evaluate toxic effects.
Results The Folin-Ciocalteau assay showed higher concentration of polyphenols in MGM3 which was used for cell treatments. Increasing doses of MGM3 significantly inhibited AU565-PAR growth. Furthermore, MGM3 induce apoptosis and early apoptosis shown by high percentage of annexin and 7AAD positive cells. No significant effects on viability and mortality of PBMCs from healthy donors was detected. In addition, untreated cancer cells showed high percentage of DCFDA-positive cells while the treatment with MGM3 induced ROS decrease.
Discussion and conclusion Many events can contribute to progression of cancer, including inflammatory and oxidative processes. In this regard, it is necessary to find substances that target tumor cells and that have both an antiproliferative and proapoptotic effect but also anti-inflammatory and antioxidant capacity. The use of plant compounds purified by HC could be optimal due to their low toxicity and fewer side effects, allowing their use in adjuvant cancer therapies. This study provides preliminary evidence to support that treatment with MGM3 obtained by HC decreased breast cancer cells viability and induced apoptosis without any dangerous effect on healthy donor cells, suggesting its possible use in future antineoplastic therapies.

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A POTION OF ETERNAL YOUTH: THE ANTI-AGING EFFECT OF VOGHERA SWEET PEPPER

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Aging and its related disorders are the main important issues nowadays and the first cause of this physio-pathological condition is the overproduction of reactive oxygen species, which leads to oxidative stress. In this regard, one of the molecules that act as an anti-oxidant mediator is Ascorbic acid and its role as an anti-aging factor is clearly well described in the literature. Our previously published results clearly demonstrated that, Capsicum annuum L. var. Peperone di Voghera (VP), a peculiar type of pepper cultivated by a limited number of producers in the province of Pavia and Alessandria, has a high nutritional value and is particularly rich in ascorbic acid. Based on these data, the anti-aging effect mediated by the high levels of vitamin C detected in the extract of edible part of VP, compared to another important Italian variety, i.e. Carmagnola pepper (CP), was evaluated in in vitro model of both young and old Normal human diploid fibroblasts (NHDF) using: (i) phase contrast microscopy, to investigate the possible morphological alteration, (ii) flow-cytometry, focusing our attention on cell cycle, and (iii) immunocytochemistry, evaluating the modification/alteration of the expression levels/cellular localization of specific markers of proliferation and aging, i.e. PCNA, p16 and p53. Using phase contrast microscopy, we first observed that VP may help cells to maintain physiological morphology, compared to CP-treated cells. Parallelly, cytofluorimetric analyses revealed that pepper extract led to an increase in G2 events, probably linked to a consequent increase in mitotic events. The hypothesis of an increase in mitotic events, following pepper treatment, was also supported by the enhancement of PCNA expression levels observed in old fibroblasts after treatment, corroborating the idea that this extract could recover the young phenotype in adult fibroblasts. Lastly, the analyses of p16 and p53 expression levels confirm the anti-aging effect of our extracts. Based on these results, we may suppose that VP is able to preserve physiological phenotype in young NHDF, modulating several molecular mechanisms involved in aging and regulating the expression levels of both proliferation and aging markers, leading to the partial recovery of “young-like” phenotype in old fibroblast.

GLIOBLASTOMA MULTIFORME: STUDY OF CELL ALTERATIONS DUE TO PLATINUM COMPOUNDS AND PHYTOTHERAPY IN T98G AND U251 HUMAN GLIOBLASTOMA CELL LINES

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Glioblastoma Multiforme is the most common and lethal primary brain tumor in human, remaining among the most difficult cancers to treat. After standard therapies, with surgical resection, Temozolomide and radiotherapy, the development of resistance is almost inevitable. Alternatives could be represented by platinum analogues, such as Cisplatin and Carboplatin, which however shows important side effects. To overcome this limitation fourth-generation platinum compounds were synthesized, one of them is Pt(IV)Ac-POA, (OC-6-44)-acetate-diamine-chloride(2-(2-propinyl)octanoate)-platinum(IV), a prodrug having an Histone-3-DeAcetylase-Inhibitor as axial ligands. Moreover, new compounds of plant origin are increasingly seen as potential sources of benefits in oncological treatments, indeed their capability to interfere in the metabolism of reactive oxygen species (ROS), in synergy with new chemotherapy could give patients an improvement in quality of life.
Aims: Here, we evaluated the effect of platinum-based compounds and a medicinal blend supplement (Agaricus blazei, Cordyceps sinensis, Grifola frondose, Lentinula edodes and Ganoderma lucidum) on the cell cycle and the morphology of T98G and U251 glioblastoma resistant cells.
Methods: Flow cytometric analysis of sample stained with propidium iodide was adopted to evaluate the possible therapeutic effects of the studied compound.
Results: Our data revealed that an alteration of the histograms representing cellular DNA content after the different treatment in both cell lines, suggesting promising compounds-induced cell cycle modification, such as the increment in the number of events in the Sub-G1 phase, the reduction of S phase amplitude and drug specific cycling blocks.
Conclusions: Due to the efficiency of the studied molecules on the cell cycle, this work offers interesting hints for future new therapies to overcome drug-resistance issue and off-target cytotoxicity in Glioblastoma Multiforme treatment.

PROAPOPTOTIC EFFECT OF THREE DIFFERENT SPECIES OF MORINGA OLEIFERA LAM. LEAVES EXTRACTS ON THP-1 CELL LINE

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BACKGROUND

The University of Rome "Tor Vergata" with UNESCO interdisciplinary chair in Biotechnology and Bioethics have grown, over the years, a tradition on teaching and North-South Biotechnology Transfer. Moringa oleifera Lam. (MO) is one of the most distributed species of Moringaceae family which is also widely used in African traditional medicine. This plant has a high nutritional value for its primary metabolites and essential nutrients like vitamins, proteins, and amino acids. It is also an important source of many bioactive compounds like carotenoids, polyphenols, and glucosinolates that have been identified in almost all parts of the plant, especially in leaves and seeds. MO leaves are the most widely studied and they have shown to be beneficial in several chronic conditions, including hypercholesterolemia, high blood pressure, diabetes, insulin resistance, non-alcoholic liver disease, cancer, and overall inflammation.Considering the emerging role of chronic inflammation as a determinant in the progression of major degenerative diseases typical of modern society and the use of MO. leaves (MO-L) as a natural supplement in traditional medical approach, we investigated the ability of three different species of MO Aqueous Extracts Leaves (MOEL) to modulate the inflammatory response in an in vitro model.

MATERIAL AND METHODS

Plant material, extract preparation and cells treatment. The MOEL was prepared according to an African traditional method. The human monocytic cell line THP-1 was used, and the cells were treated for 72 hours with different Fresh Weight Concentration (FWC) of MOEL (1/2,5/5 mg/mL).
THP-1 were stimulated or not with 20 ng/mL LPS and treated with 1 mg/mL of MOEL FWC at different time point (0/3/6/12/24 hours).
Cell death/viability. Absolute number of dead and live cells was evaluated using the trypan blue exclusion test (Euroclone).
Apoptosis assay. Apoptosis was assessed through flow cytometry analysis, using a Cytoflex (Beckman Coulter), on isolated nuclei stained with propidium iodide.
Immunostaining and intracellular flow cytometry analysis of cellular proteins. After 3, 6, 12, and 24 hours after MOEL treatments, THP-1 cells were harvested, fixed and permeabilized with Saponin. Intracellular expression of Bcl2 protein and pro-inflammatory cytokines was evaluated in by Cytoflex flow cytometer using: anti-human Bcl2-FITC; : TNF-α - PE, IL-8 – PerCP-Cy5.5, IL-1β – AF647 , IL-10 – Pe-Cy7, IL-6 – Pacyfic blue.
Results: The treatments with the two of three different MOEL induced a significant reduction of proliferation and induced apoptosis, in a dose dependent manner, on THP-1 cell line while is possible to observe a different decrease of the proliferation for the third extract. Moreover, THP-1 cells stimulated with LPS, shown a significative decrease of TNF α and an increase of IL -10. **Conclusion:** Different studies have been performed to confirm the beneficial effects of MO leaves on humans. The aim of this study was the evaluation of the biological effects of MO on human tumor cell line and study the cytokines expression after the treatment. Our studies showed that these three extract are involved in a decrease of cell proliferation and in decrease of the apoptosis. In addition, with regard to cytokine expression, we were able to ascertain an increase in the anti-inflammatory cytokine IL-10 and an increase in TNF α, that has always been associated with inflammation, leading us to think that there may be a potential anti-inflammatory effect in the three.

EFFECTS OF MORINGA OLEIFERA LAM. MICROVESICLES ON ENDOGENOUS MIRNAS INVOLVED IN TUMORIGENESIS IN HELA CELL LINE

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Background. Cervical cancer (CC) is one of the most frequent cancers in women worldwide. The epithelial-mesenchymal transition (EMT) and TGF-β production, are processes associated with different tumorigenic processes. Specific human miRNAs (hsa-miRs) involved in these processes were identified, becoming important diagnostic and prognostic markers, and potential therapeutic targets. In parallel, plant microvesicles (MVs) could modulate host gene expression under pathological conditions, restoring the regulatory activity of endogenous miRNAs lost in cancer. In our previous studies, the MVs from Moringa oleifera Lam. were characterised, and demonstrated the ability to differentially regulate proliferation and apoptosis in healthy and tumour cells. In the present study were investigated the effects of MOES-MVs on tumorigenesis, EMT and on the modulation of has-miRs profile in CC-derived HeLa cells. **Methods.** MOES-MVs were isolated and characterized for size using a Gigamix-FSC&SSC Plus kit. HeLa cells were treated with MOES-MVs purified from MOES for 72hrs. Cell viability was assessed by Trypan Blue exclusion test and apoptosis through flow cytometry analysis on isolated nuclei stained with Propidium Iodide. Mitochondrial activity assay was assessed using two assays: MitoProbe JC-1 assay, and MitoTracker Red (CMXRos) according to manufacturer's instructions. The analysis was assessed using CytoFlex CytExpert software. RNA extraction and reverse transcription of HeLa cells and of cervical swab residual samples of HPV and PAP test negative (CSRS) cells were performed by TRIzol and by cDNA Reverse Transcription Kit. Through the Human Cancer Pathway RT2 Profiler PCR Array the expression of 84 hsa-miRs were analysed in CSRS, and in HeLa treated or not with MOES-MVs. **Results.** The treatment with plant miRs was able to modulate proteins involved in EMT. It reduced the expression of TGF-β and significantly inhibited cell motility and viability, with a significant increase of apoptotic events. Among the 84 hsa-miRs analysed, 42 resulted up-regulated, 25 down-regulated, 7 had stable expression, and only one was not detectable, in HeLa cells with respect to CSRS. MOES-MVs significantly modified this profile: the treatment reduced the overexpression of 14 miRs, decreased the expression of 10 miRs, and restored the expression of 11 miRs, respect to the control. Moreover, 6 up-regulated miRs in HeLa cells respect to CSRS, resulted undetectable after the treatment. Among the 25 down-modulated miRs, the MOES-MVs induced the over-expression of 2 miRs, whereas 14 miRs resulted undetectable, and restore the expression of 2 miRs. **Conclusion.** Our results pave the way for the development of new potential therapeutic approaches based on plant MVs for contrasting human cervical cancer modulating the endogenous miRs and the pathway involved in apoptosis and EMT, even in the form of adjuvants to classic treatments for limiting their side effects.

Steem Cells and Cell Therapies

16-COLOR MULTIPARAMETRIC IMMUNOPHENOTYPING TO DETERMINE IDENTITY, PURITY, AND MATURATION OF SPECIFIC MULTIVIRUS T CELLS EXPANDED IN VITRO FOR CLINICAL USE

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Background: Our group is developing an advanced therapy medicinal product (ATMP) based on polyclonal multivirus specific T cells (MVT-cells) for autologous and allogeneic use.
The T lymphocytes are stimulated simultaneously by EBV, CMV, BK polyoma virus and Adenovirus peptides mix to generate virus-specific T cells. The efficient expansion of MVT-cells in a closed-system is an active challenge for our cell factory laboratories. Cell phenotype identity and activity/specificity as well as variability in cell yield are common pain-points when transitioning from research protocols (i.e. flask or well plates) into closed manufacturing systems. The successful viral antigen-specific T cell GMP production in innovative cell culture devices can potentially repopulate distinct T-cell subsets in vivo after infusion. Studies suggest that cells derived from the central memory compartment may be important for the long-term persistence of adoptively transferred MVT- cells.
Materials and methods: To determine the identity, purity and maturation of virus-specific T lymphocytes, we performed immunophenotypes by flow cytometry using a BD FACS Celesta SORP 4 lasers 16 color 18 parameter cytometer with FSC and SSC (forward and side scatter) using the following antibodies: CD45, CD3, CD4, CD8, to identify T lymphocytes; CD14, CD19, CD16, CD56 to determine the purity, quantifying respectively any residual contamination of monocytes, B cells and NK cells; CD45RA, CD62L, CCR7, to determine T cell maturation; HLA DR for T cells activation, CD25, CD127 for regulatory T cells, TCR γδ for γδ T cells.
Results: Optimizing cell expansion in G-Rex (M series) at small-scale is key to achieving efficient cell expansion. We analyzed, beyond specificity and activity of the expanded MVT-cells, the immunophenotypic characteristics at time zero (T0) and after 8-11 days in culture (T1). The increase of percentage of CD4+ and CD8+ MVT-cells population in G-Rex 10 M is comparable to the classic 24-well cell culture system at T1. In addition, expression of naïve cells (CD62L+CD45RA+) decreased, while CM T cells (central memory CD62L+CD45RA- cells) and EM T cells (effector memory CD62L-CD45RA-) increased at T1 compared to T0 in both culture systems. We also quantified exhaustion identifying the T cells TEMRA (terminal effector memory CD45RA+CD62L) separately for CD4+ and CD8+ subpopulations.
Conclusion The implementation of the described 16-color panel allowed to determine a complete immunophenotyping of expanded MVT cells in innovative in vitro culture systems (10M G-Rex series) analyzing only one small sample (about 106 cells). This is a valid experimental approach to evaluate identity, purity but also different T cell subpopulation markers as predictor of potential efficacy in vivo.

CHARACTERIZATION OF HETEROGENEOUS STEM-LIKE CELLS COEXISTING IN SINGLE GBMS

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Introduction: Glioblastoma multiforme (GBM) is an extremely aggressive brain tumor, characterized by remarkable intratumoral genetic and phenotypic heterogeneity. Several studies have shown that GBM cells are hierarchically organized, with a subpopulation of cancer stem cells (GSCs) at the apex, responsible for tumor initiation, progression, resistance to conventional therapies and recurrence. GSCs are typically isolated from patient biopsies using a culture technique that produces “neurospheres” (NS) in serum-free medium supplemented with EGF and FGF2. The current methodology mostly relies on tissue samples of limited size, which may not fully capture the entire range of GBM heterogeneity (including subclones with different RTK expressions that can be located in separate GBM regions). Moreover, standard culture conditions may select for a limited number of subclones that have the highest fitness under those conditions, potentially leading to an incomplete picture of the multiple subclones composing the tumor. We developed a new methodology for GSC isolation aimed at better capturing GBM heterogeneity.
Material and Methods: We collected a cohort of human GBMs surgically removed as ultrasonic aspirates (UA; n=31), virtually allowing the recovery of the entire tumor mass. We identified UA GBM cells (by discriminating the CD45neg/CD56pos population of cells of neuroendocrine origin from residual CD45pos leukocytes), and we established multiple parallel cultures of NS (“NS families”) by applying different positive selective pressures, represented by different cocktails of growth factors (EFG, FGF2, PDGFB, and HGF). Four representative NS families were characterized by transcriptional profiling and by flow-cytometry for expression of Receptor Tyrosine Kinase (RTK) and markers associated with different transcriptional profiles.
Results and Discussions: Starting from 31 patients, we derived 19 different GSC families, defining “family” the set of NS derived from a single GBM, and “family member” each NS culture belonging to the same family. Within each family, the members, although sharing the same driver genetic lesions are highly transcriptionally and biologically heterogeneous. Interestingly, we observed that features coexisting in original GBMs, such as concomitant expression of multiple RTKs, were segregated in different GSC family members, in association with different GBM transcriptional profiles and biological properties.
Conclusion: We designed a new methodology to derive multiple GSCs from single GBMs, each segregating distinctive molecular and biological aspects coexisting in the original tumor. RTK expression is consistently associated with different GBM molecular subtypes.

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ANALYSIS OF THE INTERACTIONS BETWEEN T CELLS AND NON-VIRAL NANOVECTORS FOR GENE DELIVERY BY FLOW CYTOMETRY

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Nowadays one of the main challenges in adoptive cell therapy is the delivery of nucleic acids into T cells. The principal effective ways of transfection are based on viral vectors and electroporation, but both present some issues as high costs of production, oncogenic insertions and high cell mortality.
The aim of our research is to develop a nanoparticle-based method to genetically engineer T cells. In this context, we analyse how T cells interact with different kinds of nanoparticles (NPs) and their components through flow cytometry.
To achieve this aim, PBMCs are collected from healthy donors. T cells are isolated and then, stimulated and activated for proliferation and expansion. This step is accomplished by the proper treatment with antibodies, as well as by a well-balanced combination of cytokines in the medium.
After activation, T cells are treated with different NPs and analysed through flow cytometry to assess cell phenotypes through measurements of intrinsic cellular properties: cell size and shape by forward-angle light scatter (FSC) and cellular granularity and morphology by side-scattered light intensity (SSC). In addition, specific cellular staining is performed to analyse the change in different sub-T cell populations, such as the biocompatibility of tested nanoformulations.
Still now, different nanomaterials are been tested on activated T cells evaluating the morphological changes through flow cytometry. Results highlight that several NPs induce dimensional and internal complexity changes, thus resulting cytotoxic, while single constituent of NPs don't entail any morphological changes. This evidence reveals the role of nanostructuration in conferring biocompatibility/cytotoxicity properties to nanoformulations intended for genetically engineering of T cells.
In conclusion, we are working on identifying principles of design and rules for the production of NPs that result in high biocompatibility and high transfection efficiency and flow cytometry represent a key analysis technique for the interaction analysis of nanomaterials with T cells and other components of immune system.
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DNA DAMAGE IN CIRCULATING HEMATOPOIETIC PROGENITOR STEM CELL: A POSSIBLE MARKER OF FRAILTY?

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Frailty is a clinical syndrome closely linked to advanced age. It can be defined as a state of increased vulnerability, resulting from severe decline of several physiological systems and in a reduced capacity to compensate for external stressors. Although it is an intermediate and potentially reversible state, this syndrome predisposes to the development of disability and other adverse health-related outcomes, including mortality.
Although there are no molecular processes or biological markers able to identify a frail phenotype, inflammation is a potential pathophysiological change that may be closely related to frailty.
We thus hypothesize that the increase in oxidative stress, related to this inflammatory state, can be a major cause of DNA damage accumulation in aged cells and could be implicated in frailty.
Given these premises we characterized the oxidative status of frail, pre-frail and non-frail populations, highlighting significant increases of auto-oxidation of oxysterols, as well as of 8-OH-dG, which is produced by the oxidative damage of DNA, in frail plasma compared to pre-frail and non-frail plasma.
Moreover, we analysed the DNA damage in peripheral blood mononuclear cells (PBMC) and in their subpopulations, especially in circulating hematopoietic progenitor stem cell (cHPSC), by assessing the level of histone H2AX phosphorylation at Ser 139, the early event in response of DSB.
We found a significant increase of cells with DNA damage in cHPSC and PBMCs in frail population compared to others.
This increment of DNA damage in cHPSC could be a suggestive signal of organism impairment that precedes the evident frailty in older people.
In addition, we can hypothesize that progression of frailty may be attenuated throughout specific drugs that act on preventing DNA damage or on removing the damaged cells.

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COVID-19

A RAPID AND SIMPLE MULTIPARAMETER ASSAY TO QUANTIFY SPIKE-SPECIFIC CD4 AND CD8 T CELLS AFTER SARS-COV-2 VACCINATION

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After more than four years, the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused, over 760 million confirmed cases and over 6.8 million deaths worldwide [1]. mRNA vaccines for COVID-19 are used to induce humoral and cell-mediated immunity, but SARS-CoV-2 can spread from cell to cell without exposure to the extracellular environment [2], limiting the role of NAb in the immune response against this virus. For this reason, T cells can be essential mediators of the protective host response to SARS-CoV-2 infection.
Here we describe a simple flow cytometric test to detect and quantify Spike-specific CD4+ and CD8+ T cell responses induced by vaccination in healthy donors and in subjects with B cell compart impairment, in which antibody response is absent due to primary immunodeficiencies or therapy.
This assay relies on the simultaneous expression of three cytokines (IL-2, IFN-γ and TNF-α) after 18 h of stimulation in vitro with a pool of lyophilised peptides covering the immunodominant sequence domains of the spike glycoprotein of SARS-CoV-2.
The enrolled subjects, all vaccinated with Pfizer, were divided into four groups; the first three groups were composed of healthy donors with different titers of SARS-CoV-2 NAbs at the time of venipuncture. The fourth group comprised patients without functional B cells and no NAb in peripheral blood.
We detected and quantified memory T cell immune responses against SARS-CoV-2 evocated by vaccination in all the groups, irrespective of the humoral response. Furthermore, we identified TNF-α as the main cytokine produced by T memory cells, after antigen-specific stimulation in vitro, that could be considered, other than IFN-γ, an additional biomarker of induction of T memory cells upon vaccination. Our study shows that vaccination gives a robust cell-mediated immunological memory against spike protein antigens, independently from the titer of NAb, meaning that the T cell-mediated specific immune response against SARS-CoV-2 can develop independently from the B cell response [3]. This finding highlights the importance of the cell-mediated immunity against SARS-CoV-2 not only in healthy people but also in B cells compromised subjects, where T cells have a predominant role in the protection against SARS-CoV-2.

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CHARACTERIZATION OF CD169 EXPRESSION ON BLOOD CELLS AND CIRCULATING MICROVESICLES AS A POTENTIAL MARKER IN DEVELOPMENT, AND PROGRESSION OF COVID-19 AND POST-ACUTE SEQUELAE

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Background: An elevated inflammatory response and immune dysregulation are the main consequences of SARS-CoV-2 infection and characterize COVID-19 disease. This dysregulated inflammatory state persists even after infection, generating the post-acute sequelae of SARS-CoV-2 infection (PASC) characterized by neurological, psychiatric, and systemic manifestations. The identification of new biomarkers that can characterized COVID-19 and predict its long-term effects are needed. CD169+ macrophages play an important role in viral infections and recently has been demonstrated that CD169 was strongly overexpressed in the blood of COVID-19 patients (COV). Aim: Based on our recent evidences supporting the close implication of CD169 expression in COVID-19, the aim of the present study was to extend the evaluation of these potential biomarkers also in PASC, to identify possible associations also with important alterations following the infection and potentially underlining the post-infection sequelae. The study also aimed to investigate the CD169 in association to the immune dysfunction mirrored at circulating level, in particularly focusing on plasma circulating microvesicles (MVs). **Materials and methods:** Flow cytometry was used for the evaluation of the median fluorescence intensity ratio of CD169 between monocytes and lymphocytes (CD169 RMFI) in blood samples from 60 COV, 33 PASC and 43 healthy donors (HD). Leukocytes subpopulations and MVs were characterized for HLA-DR+CD169 proteins expression. Serum inflammatory markers (IL-1β, CXCL10, IL-8, I-CAM, V-CAM) were assessed by Ella Automated Immunoassay System. The different markers analyzed were associated with clinical and biochemical parameters in COV and PASC. **Results:** CD169 RMFI was found significantly higher in COV than in HD and PASC. Among the leukocyte populations, monocytes showed a significantly higher percentage of HLA-DR+CD169+ in COV and PASC than in HD and correlated with CXCL10 and number of platelets. In plasma, the percentage and number of
HLA-DR+CD169+ circulating MVs were significantly elevated in COV and PASC compared to HD and correlated with coagulation factors. **Conclusions:** These data sustain a potential role of MVs as a contributing factor to COVID-19 disease and post-infective chronic inflammation. This scenario opens new possibilities for studying the use of CD169 at cellular and MVs level as markers and potential cofactors either during acute infection or in post-infection sequelae. The characterization of the contribution of MVs using CD169 marker could clarify the complexity of COVID-19 and evaluate their involvement in the long-term complications seen in PASC.

T CELL RESPONSE TO VIRAL PEPTIDES OF VACCINATED AND RECOVERED FROM MILD COVID-19 SUBJECTS MAY CONTRIBUTE TO DEFINE HOST-PATHOGEN RELATIONSHIP

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Introduction

Many studies show that SARS-CoV-2 natural infection as well as active vaccine immunization induce humoral and cellular immune responses, recruiting both antibody producing B cells and antigen-specific T cells. Thus, it is crucial to assess the levels of protection generated by SARS-CoV-2 natural infection or vaccine, by measuring T-cell responses that may complement currently in use antibody testing to find correlates of protection. Epitopes recognized by CD8+ T cells can encompass both structural and non-structural proteins, since both types of proteins can be endogenously processed by infected cells.

The aim of the present study was to identify potential novel peptides of interest derived from Spike and some viral non-structural proteins. In addition, we intended to correlate T cell response to peripheral immuno-phenotype as well as to anagraphic/clinical characteristic of the enrolled subjects, in order to better define host-pathogen relationship.

Method

We firstly selected some HLA-A * 02: 01 restricted epitopes derived from the Spike and few non-structural proteins (Nsp1-2-3-16), by using the predictive bioinformatic algorithm (NetMHCpan EL 4.1). We thus obtained 15 poorly investigated peptides for Spike (including 4 mutated peptides for Delta and/or Omicron variants) and 3 peptides for each of the non-structural proteins, with promising prediction scores in terms of immunogenicity. Afterward, we further tested the immunogenicity by IFN-γ ELISPOT assay on cryopreserved PBMCs isolated from peripheral blood of 14 vaccinated and recovered from mild COVID-19 HLA-A * 02: 01 healthy subjects. Furthermore, subjects' anagraphic/clinical data were collected by a survey questionnaire, while leukocytes immuno-phenotype was assessed on whole blood samples of the enrolled subjects by means of standardized multiparametric flow cytometry panels.

Results

The poorly studied selected peptides induced low and variable immune response on subjects' PBMCs, except one of them, named LA9 (a Spike protein fragment) that showed a good response rate (50%).

Despite the small number of enrolled subjects, we found significant association among peptide T cell response and certain T memory subset frequencies as well as some anagraphic/clinical features. Indeed, individuals with high cellular response are characterized by young age, intensive sport activity, low frequency of central memory T cells and a low number of symptoms during the infection.

Conclusion

Based on our findings, we can reckon that T cell response studies can efficiently contribute to better define interaction between host and viral pathogen. Nevertheless, further analysis on a larger number of subjects are needed to confirm our results.

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