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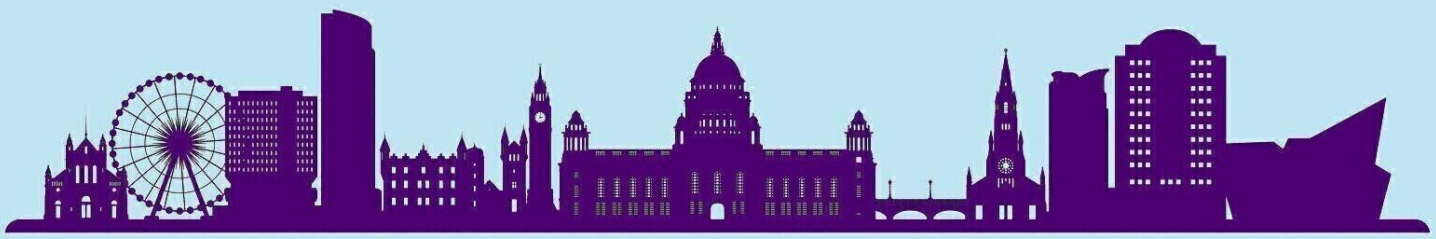

ESCCA 2022

BELFAST, NORTHERN IRELAND, UK

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ABSTRACTS

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PLENARY SESSION 1

PLE01-01

CAR-T CELLS AND BEYOND

Dario Campana

T-lymphocytes genetically modified with chimeric antigen receptors (CARs) have produced dramatic clinical responses in patients with acute lymphoblastic leukemia (ALL), non-Hodgkin lymphoma and multiple myeloma. Because of their effectiveness, CAR-T cells are being progressively incorporated in frontline therapy. In B-cell malignancies, CD19 represents an excellent target for CAR-T cell therapy because of its wide expression. Among normal cells, it is only found in B lymphocytes and their progenitors, whose depletion is not life-threatening. In patients with ALL, monitoring of minimal residual disease (MRD) is essential to identify those who have a higher risk of relapse with standard therapy and may benefit from CAR-T cells. MRD monitoring by flow cytometry also allows to determine whether a subset of ALL cells lacks CD19 expression and, if so, whether these cells express other targetable antigens. Moreover, detection of rare events by flow cytometry is applicable to the monitoring of CAR-T cells post-infusion as well as to the presence of B lymphocytes in peripheral blood and of normal B-cell progenitors in bone marrow. These measurements can provide information on CAR-T cell function. The systematic use of these strategies should ultimately reduce the prevalence of relapse after CAR-T cell infusion due to the emergence of CD19-negative clones and allow more timely intervention if the performance of the infused cell product is suboptimal. These issues will be discussed together with approaches to target other hematologic malignancies and solid tumors, as well as technologies to improve and expand the functionality of immune cells.

PLE02-01

MONITORING T-CELL IMMUNITY IN COVID-19 – TRANSLATIONAL STUDIES

Nina Babel

Infection is the fourth leading cause of death in general population and second leading cause of death in patients with chronic diseases such as chronic kidney diseases or diabetes. Sufficient function of both arms of immune systems - humoral (antibody) and cellular (T cell-driven) immunity that required for the prevention of pathogen entry and clearance infectious pathogens, respectively, can control infection.
The current presentation will focus on the formation of SARS-CoV-2-specific T cell immunity during natural infection or following vaccination. In context of natural infection, the role of pre-existing cross-reactive T cell immunity as well as different SARS-CoV-2 proteins will be discussed. I will present functional and phenotypic characteristics of SARS-CoV-2-specfic T cells associated with different stages of COVID-19 that might allow personalized therapy. A special focus will be on patients with chronic kidney disease - the most vulnerable patient population. In context of COVID-19 immunization, immunogenicity of COVID-19 vaccines as defined by vaccine-induced antibody and T cell response will be demonstrated in different patient cohorts including immunocompetent and immunocompromised patients. The role of immune monitoring for the vaccination recommendations in a risk population such as immunocompromised patients will be demonstrated and discussed.

PLE03-01

A NOVEL CHUNK-FOR-POOLING MACHINE LEARNING MODEL TO DETECT MRD IN AML WITH FLOW CYTOMETRIC DATA

Yun-Chun Lin [1], Jeng-Lin Lin [2], Yu-Fen Wang [3], Paul K. Wallace [4], Bor-Sheng Ko Ko [5], Teng-Jen Chang [1], Yun-Chun Huang [1], Chi-Chun Lee [2]

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Introduction: In the treatment of acute myeloid leukemia (AML) patients, detection of measurable residual disease (MRD) is critical in guiding effective treatment and predicting complete remission. Manual analysis, however, of flow cytometry (FCM) data for AML MRD detection is complex, requiring significant expertise and time consuming. This study proposed a novel machine learning (ML) framework that addresses the potential caveats of using downsampling for computing memory to develop an automated classification model for detecting MRD in AML. Our approach expedites detection and triages processes for AML prognostic care.
Method: We analyzed FCM datasets for 1,040 bone marrow specimens collected at Roswell Park Comprehensive Cancer Center between 2015 to 2020, stained with their 8-color AML MRD panel, and acquired on a BD FACSCanto™ II. Presence of MRD was based on the Residual Disease Percentage (RDP). There were 691 (66.4%) MRD-negative specimens (RDP <0.001) and 349 (33.6%) MRD-positive specimens (RDP between 0.001 and 97.7). Based on histology, 711 (68.4%) were normocellular, 117 (11.3%) specimens were hypercellular, and 212 (20.3%) specimens were hypocellular. We leveraged a chunk-for-pooling framework to train an ML classifier that distinguished MRD-negative and MRD-positive specimens. The FCM datasets for each specimen were chunked into small equal-sized segments. Segments preserving immunophenotype characteristics were pooled together to construct specimen-level representations which fed a deep neural network to train the AML MRD classifier. We applied this approach on the whole dataset (n=1040) and the normal-cellularity sub-dataset (n=711).
Result: The model achieved an area under the ROC curve (AUC) of 88.5%. Incorrect predictions were observed more frequently on specimens with low cellularity (20.5%, n=160) than on normal-cellularity specimens (11.5%, n=30). The model on the sub-dataset outperformed the whole dataset (AUC=89.1%). Incorrect predictions were more probable on abnormal cellularity specimens (19.2%, n=99) than on normal-cellularity specimens (10.3%, n=20).
Conclusion: We demonstrated here the potential of the chunk pooling model to support automated AML-MRD detection of large data files. The model performance was even better when the targeted dataset was constrained to specimens with normal cellularity. Overall model performances could be affected by parameter (e.g., chunk sizes) selections. Immunophenotypes captured by chunks can be helpful for explaining the decision made by the classifier and provide a robust result for clinical use. The relationship between cellularity, RDP, and model performances is worth further investigation.

PLE03-02

STANDARDIZATION OF 13-COLOR HIGH-SENSITIVITY MEASURABLE RESIDUAL DISEASE (MRD) ASSESSMENT IN B-CELL LYMPHOBLASTIC LEUKEMIA (B-ALL) TREATED WITH NOVEL ANTI-CD19 IMMUNOTHERAPIES

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Nilesh Deshpande, Harshini Sriram, Sweta Rajpal, Gaurav Chatterjee, Nikhil Patkar, Chetan Dhamane,
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Introduction: Measurable residual disease (MRD) assessment is a powerful tool for monitoring therapeutic response. CD19 is a principle gating B-cell marker for flow cytometric B-cell lymphoblastic leukemia (B-ALL) MRD assessment. However, the inclusion of immunotherapies targeting CD19-antigen (e.g. blinatumomab, CD19 CAR-T, etc.) for the treatment of relapsed/refractory B-ALL has led to the loss of CD19 on B-cells and residual B-ALL blasts and thus, made it challenging to gate them. Herewith, we share our experience with 13-color high-sensitivity MRD assays standardized for therapeutic response monitoring of B-ALL post-antiCD19 immunotherapies.
Methods: We standardized a 13-color flow cytometric MRD assay using antibodies against CD10, CD19, CD20, CD22, CD24, CD34, CD38, CD45, CD73, CD81, CD86, CD123, and CD304. The samples were processed using a bulk-lyse-wash method and a minimum of 4 million cells were acquired on FACS LSR Fortessa and data was analysed using Kaluza 2.1v software.
Results: We incorporated 3 new markers i.e. CD22, CD24 and CD81 for gating B-cell precursors to our existing B-MRD panel published earlier. We compared results of CD22-BV786 versus CD22-PE, CD24-PerCP-Cy5.5 versus CD24-BV650 and CD81-APCAlexa750 versus CD81-ECD. Among them, CD22-PE, CD24-BV605 and CD81-APCAlexa750 were found superior. In addition to identifying MRD, some of the markers were found useful in identifying other hematopoietic/non-hematopoietic cells that can easily interfere with MRD assessment leading to interpretational errors. These included 1. the most-primitive B-cell precursor (stage-"0" hematogones) which are CD22(+)/CD19(-)/CD10(+/-)/CD34(++)/CD24(-)/ CD81(++)/ CD38(++), 2. basophils with CD22(+)/CD19(-)/CD10(-)/CD34(-)/CD24(-)/CD81(++)/ CD38(++)/CD123(++) and 3. mesenchymal stem/stromal cells CD22(-)/CD19(-)/CD10(+++)/CD34(++)/ CD24(-)/CD38(-)/CD81(++)/CD304(++)/CD123(-). New gating strategy helped to identify stage-0 hematogones in 18, stage-1 in 19 cases and stage 2 hematogones in 16 samples. We studied 10-control samples (4 T-ALL MRD & 6 AML MRD), 47 bone marrow samples from 18 B-ALL children treated with either blinatumomab (n=13) or antiCD19-CAR-T therapy (n=5), and 18 samples from 13 patients screened for antiCD19 immunotherapy. MRD was detected in 12/47 samples evaluated post- antiCD19 immunotherapy (range: 0.0002% to 36.7%) and two of them showed negative CD19 expression. Of 18 samples screened for antiCD19 immunotherapy, 6/18 samples were MRD-positive (range: 0.0007-48.78%).
Conclusions: We successfully developed a new gating strategy using a 13-color high-sensitivity B-ALL MRD assay for therapeutic monitoring of antiCD19 immunotherapy response. This approach also allowed distinguishing stage-0 hematogones and other marrow cells interfering with MRD assessment.

PLE03-03

CLINICAL RELEVANCE OF IMMUNOPHENOTYPIC SIGNATURE OF MYELOBLASTS AND PROPORTIONS OF HEMATOGONES AND MONOCYTES IN JUVENILE MYELOMONOCYTIC LEUKEMIA

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Introduction: Juvenile myelomonocytic leukemia (JMML) is a rare myelodysplastic/myeloproliferative neoplasm overlap syndrome of children with dismal outcomes. The data on immunophenotypic findings in JMML is scarce and its clinical relevance in the diagnosis and management of JMML is not adequately studied. We report immunophenotypic findings in bone marrow (BM) samples in 33-cases of JMML.

Methods: A 13-color five-tube antibody-panel including antibodies for CD1a, CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD16, CD19, CD20, CD22, CD25, CD33, CD34, CD36, CD38, CD42b, CD45, CD56, CD64, CD117, CD123, cytoCD79a, cytoCD3, cytoMPO and HLADR was studied for abnormal/asynchronous antigen expression-patterns in progenitors and maturing granulocytic/monocytic cells. The immunophenotypic findings were correlated with genetic aberrancies/mutations.

Results: We included 33 children (age: median-3years, and range-0.5-14years; M:F-2.3:1) diagnosed with JMML. Genetic aberrancies/mutations included monosomy-7(27.3%), PTPN11 (27.3%), NRAS(21.2%), KRAS(15.1%), NF1(9.1%) and CBL(3%) gene mutations. Median (range) of total CD34+progenitors, total hematogones, and myeloblasts were 6.41%(1.34%-15.9%), 0.83% (0.0-33.66%), and 4.57%(0.14%-15.9%) respectively. Within CD34+progenitors, median (range) of CD34+hematogones was 5.2%(0.0-59.67%).

CD34+myeloid blasts demonstrated a unique immunophenotypic signature with heterogenous/ subset CD7 (97% cases), CD2 in subset (69.6% cases), CD5 negative (85% cases), dim-negative CD13 (69.7% cases) with heterogenous-expression of CD33, CD38 and CD117. Notably, stem-cell-like CD38dim-negative and CD34-strong positive compartment of CD34+myeloblasts revealed dim-negative expression of CD11b (78.9%), CD25 in subset (95%) and over-expression of HLADR (60.6%). CytoCD3 was positive in a small subset of CD34+myeloblasts in 50% samples (13/26) additional to CD7 expression.

Abnormal maturation-pattern of granulocytes with CD13/CD16, CD13/CD11b, CD11b/CD16, CD56+granulocytes and other aberrancies were noted in 21.4%, 36.36%, 30.3% and 21.2% cases respectively. Within total monocytes, median (range) of immature-monocytes (CD14dim-negative/HLADR-bright), classical-monocytes, intermediate-monocytes and non-classical monocytes were 10.1%(0.03%-56.9%), 46.5%(0.75-96.28%), 26.9%(0.03%-63.48%) and 6.67%(0.04%-47.96%) respectively. Abnormal maturation-pattern of monocytes with CD14/HLADR, CD13/CD15, CD56+monocytes and other aberrancies were noted in 48.5%, 54.5%, 9% and 9% cases respectively.

Interestingly, decreased proportions of hematogones (<1.57%) in all viable cells and CD34+hematogones (<1.54%) in total CD34+progenitors were strongly associated with monosomy-7 (p=0.006). Similarly, decreased proportion of classical monocytes (<41.7%) of all monocytes was strongly associated with PTPN11 mutation (p<0.001).

Median follow-up was 6.3 months (range: 0-47.8 months). 13/33 patients expired and two patients progressed to AML and MPAL(T/Myeloid) each.

Conclusion: Flow cytometric analysis provides a unique immunophenotypic signature of myeloblasts in JMML patients and is found useful for its diagnosis. The proportions of hematogones and classical monocytes also provide relevant information to predict underlying genetic aberrancies.

PLE03-04

TOWARDS A HIGH-PARAMETER SPECTRAL IMMUNOPHENOTYPING PANEL FOR PEDIATRIC LEUKEMIAS

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Introduction: Our current diagnostic immunophenotyping (IP) panel for new and relapsed pediatric leukemias consists of a multi-tube panel of ten surface and three intracellular tubes covering forty-nine discrete markers on the 8-parameter BD CantoII platform. Following lineage assignment based on the IP data, lineage-specific (B-, T-ALL, or AML) tubes are then run on the diagnostic sample to select the best markers available to follow an individual patient's response to treatment and to determine the presence of minimal residual disease (MRD) at later time points. The patient-specific MRD screening tubes containing additional lineage-specific markers are built around a backbone of four to five lineage-defining antigens unique to each disease. In the first step towards migrating our flow cytometry workflow to a high-parameter spectral platform, we are developing a comprehensive single-tube 38-parameter spectral cytometry IP panel for screening new or relapsed childhood leukemias on the Sony ID7000 Spectral Analyzer.
Methods: The current single-tube spectral IP screening panel consists of five intra-cellular markers (TdT, cyCD79a, cyCD3, cyCD42b and MPO), thirty-one surface markers (CD1a, CD2, sCD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD11c, CD13, CD14, CD15, CD16, CD19, CD20, CD22, CD33, CD34, CD38, CD45, CD56, CD61, CD64, CD71, CD117, CD123, CD133, CD235a, HLA-DR, NG2), a viability (Zombie NIR) and a nuclear marker (Draq5). Each marker in this panel is defined by a discrete fluorochrome.
Results: Iterations of the spectral panel design were tested with normal peripheral blood (NPB), leukemia patient PB and bone marrow (BM) samples covering B- and T-ALL, AML, and MPALs. We observed perfect concordance between the immunophenotyping results from the new spectral analysis and the standard CantoII IP panel. The presence of the viability marker and the nuclear marker allows accurate gating on the live-nucleated cellular component of the sample and removes the non-specific staining associated non-viable cells and cellular debris. Spectral cytometry also provides a robust mechanism to identify auto-fluorescent populations that may interfere with analysis. Indeed, with spectral cytometry, multiple discrete auto-fluorescent populations can be treated as additional parameters for spectral unmixing and can be included in the gating strategy.
Conclusions: The high-parameter single-tube spectral IP panel provides robust immunophenotype characterization of suspected pediatric leukemia samples. Our goal is to migrate the IP, and eventually MRD, analysis of our pediatric leukemia patients to the high-content spectral platform. This will provide many benefits to both our laboratory workflow and downstream analysis and will complement our molecular diagnostic strategies.

PLE05-02

QUIESCENT IMMUNE MEMORY IN BONE MARROW

Hyun-Dong Chang

A hallmark of the adaptive immune system is the establishment of immunological memory following vaccination or clearance of infection. Immunological memory provides protection when the pathogens are reencountered and can last up to a lifetime. The cellular correlates of immune memory are the memory T cells, memory B cells and long-lived plasma cells. It has been unclear for a long time, how immune memory is maintained longterm, particularly in view of the steady decline of memory cells in peripheral blood in the absence of antigen. We and others could demonstrate that cells of the immune memory against systemic antigens reside in the bone marrow, resting in terms of cell cycle, transcription and mobility. In the bone marrow, the memory cells are residing in direct contact with bone marrow stromal cells. Contact to stromal cells seems to be essential for the longterm survival of the memory cells, providing them with critical survival signals, such as activation of the PI3K signalling pathway. Thus, we challenge the dogma that memory cells maintaining longterm memory are circulating in constant search for their antigen, but rather that they rest in the bone marrow and provide protection, even when they are no longer detectable in the periphery.

PLE06-01

BIG TECHNOLOGY, BIG DATA, WHERE AND HOW ARE WE MOVING?

Dr Andrew Filby, Innovation, Methodology and Application (IMA) Research Theme Lead, Faculty of Medical Sciences, Newcastle University, UK

Over the past 10 years the field of single cell analysis (cytometry) has advanced significantly with respect to the number of parameters that can be measured on a single cell. This has been driven primarily by technologies such as single cell mRNA sequencing (scRNA-seq) whereby a relatively small number of cells can be interrogated by 1000s of parameters. This has led to significant discoveries of new cell types and cell states in human development, health and disease. While scRNA-seq is a powerful method, there are some concerns around the cost/throughput and nature of relying on mRNA alone to classify a cell. To this end fluorescence flow/mass cytometry has been used to validate, compliment and apply the findings made from scRNA-seq data both at the protein and population scale (sample throughput/cost etc.). Fluorescence flow and mass cytometry have also advanced significantly in terms of parameter space with an excess of 40 parameters possible on a per cell basis. More recently the use of oligo-tagged antibodies (CITEseq/Abseq) has allowed for truly “multi-omic” measurements to be made at mRNA and protein level on single cells adding even more capability to characterise cell types and states. Traditionally these high parameter approaches have been the domain of discovery research rather than clinical, mainly due to the cost per sample/cell, complexity of technologies/methodologies and applicable scale. As such the single biggest challenge of complex single cell data analysis has not yet been fully addressed in the clinical arena. This talks will give an overview of the “state of the nation” with regard to single cell technologies that generate “big data” and how the various approaches developed for analysing these types of data in discovery research could be applied to clinical studies.

PLE07-01

LONG-TERM MONITORING BY FLOW CYTOMETRY OF T-CELL RELATED IMMUNOLOGICAL MEMORY IN A GROUP OF IMMUNOSUPRESSED PATIENTS AFTER COVID-19 VACCINATION

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Introduction: Since very early in the SARS-Cov-2 pandemic, flow cytometry (FCM) has shown its value for revealing immunological features and consequences of COVID-19 disease. FCM also allows assessing the eficiency of COVID-19 vaccines, based on detecting antigen-specific T cells. T-cell responses are essential for protection against infectious disease through long-term immunity. For this reason, analysis of parameters related to T-cell dependent immunological memory is especially relevant to assess the efficacy of COVID-19 vaccination in immunosupressed patients.
Methods: This study included 22 immunosuppressed patients and an age- and sex-matched group of 21 controls, analyzed at days 30, 90 and 180 after complete schedule of mRNA vaccines. Circulating CD8 T cells, specific for SARS-Cov-2 Spike antigen (YLQPRTFLL epitope) were detected using an APC-conjugated HLA-A*0201 dextramer (Immudex) after PBMC isolation. For general whole blood leukocyte immunophenotyping, the DuraClone IM Phenotyping Basic Panel (Beckman Coulter) was used. Specific definition of memory-related T cell-subpopulations was performed with the DuraClone IM Phenotyping T-cell Subsets Panel (Beckman Coulter). CD4 and CD8 T cells producing IFNγ, TNFα and/or IL-2 upon whole-blood stimulation in vitro with PMA/Ionomycin were quantified with DuraClone IF T Activation Panel (Beckman Coulter). All assays were run on a Gallios flow cytometer (Beckman Coulter) and analyzed with Kaluza software (Beckman Coulter). For statistical analysis, the Mann-Whitney test was used.
Results: Regarding SARS-Cov-2 antigen-specific memory, immunosuppressed patients presented lower and more heterogeneous levels of epitope-specific CD8 T cells than controls. As for T-cell related memory markers, lower percentage of both CD4 and CD8 central memory cells was found in the immunosuppressed group. CD4 and CD8 lymphocytes of immunosuppressed patients showed a lower expression of cytokines by activated cells producing IL-2, but they exhibited a greater capacity for production of IFNγ and TNFα in the absence of IL-2 expression.
Conclusions: Our data demonstrate that antigen-specific CD8 memory is heterogeneous in both healthy and immunosuppressed individuals, but even in this group, circulating antigen-specific CD8 lymphocytes are present 6 months after RNA vaccine. As expected, the in vitro T-cell activation responses and the generation of polyfunctional T cells is lower in the immunosuppressed patients. Combining FCM assays of specific- and T-cell memory provides complementary data to conventional assays of humoral response for monitoring vaccinal responses. While our approach is too complex for routine monitoring it can be applied for personalized follow-up of high-risk patients. Sponsored by IRAS-VAC Project (Fundación Mutua Madrileña).

PLE07-02

FAST-SCREENING FLOW CYTOMETRY METHOD FOR DETECTING NANOPLASTICS IN HUMAN PERIPHERAL BLOOD

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Introduction: Nanoplastics (NPs) are plastic particles from 1nm up to 1000nm widely distributed in the environment. Plastics are reported to have negative effects in living organisms. Aquatic plastic pollution has been extensively studied though little is known about accumulation in humans. In this work, we have prospectively studied the presence of NPs in human peripheral blood in healthy individuals, newborns and a series of hematopoietic and non-hematopoietic malignancies.
Methods: Peripheral blood (PB) samples were collected in EDTA-anticoagulated tubes from healthy and non-healthy donors with informed consent. Nile Red (NR) was used to detect the most common plastics used today: low density polyethylene, polystyrene, polyethylene terephthalate, and polyamide. 100% of the organic matter was degraded by incubating 20µL of PB in the presence of 1% KOH (1mL final volume) at 60°C in a dry block for a minimum of 10 days. For fluorescence staining, 20µL of each of the degraded samples was added to deionized water (1mL final volume) and incubated with 2µL of NR 0.01mg/mL for 15 min, light-protected at RT. 200, 500 and 800nm calibration microspheres (Bangs Laboratories) were used for size-calibration on the Attune™ NxT flow cytometer (Thermo Fisher), with the H-pulse parameter and collecting violet-SSC. The IncuCyte® SX5 Live-Cell Analysis Instrument (Sartorius Stedim) was used as a real-time system to capture and analyze images of phagocytic cells over time. Fluorescent green microspheres (Count Check Beads green, Sysmex) were added for the rapid and accurate detection and quantification of *in vitro* phagocytosis.
Results: Our analysis confirms the presence of nanoplastics in human blood in more than 200 individuals, including healthy donors, newborns, and patients with acute lymphoblastic leukemia, acute myeloid leukemia, chronic lymphocytic leukemia, multiple myeloma, non-small cell lung cancer, idiopathic nephrotic syndrome, and type 1 diabetes. In general, there was good association between triplicate measurements, showing high dispersion between individuals, especially in healthy donors (n=37, median=667 events/µL, range=88-1460 events/µL). The highest levels of NPs were found in AML (n=46, median = 630.6, range=238.9-1274 events/µL), whereas the lowest were found in T1D (n=10, median=477.1, range=104.3-962.9). Fluorescence live cell imaging microscopy showed that phagocytes were unable to engulf or degrade plastic microbeads.
Conclusion: In this study, we have demonstrated the existence of nanoplastics in human peripheral blood. Flow cytometry is a highly sensitive tool to study plastic nanoparticles, with advantages over other techniques, such as Raman spectroscopy, which can resolve generally 1-2 µm particle sizes.

PLE07-03

FUNCTIONAL AND PHENOTYPICAL CHARACTERIZATION OF IFN-Γ-PRODUCING SARS-COV-2 SPECIFIC MEMORY T CELLS FROM COVID-19 CONVALESCENT DONORS

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Introduction: the SARS-CoV-2 pandemic, responsible for millions of deaths worldwide, has promoted the research in novel therapies to treat severe COVID-19 patients. Here, we analyzed the phenotypical and functional characteristics of IFN-γ-producing SARS-CoV-2-specific T-cells (SC2-ST) from convalescent donors, to determine their feasibility as an adoptive therapy strategy aimed to treat COVID-19-associated severe pneumonia in hospitalized patients.
Methods: 12 mild COVID-19 convalescent donors were recruited. The identification, quantification and phenotypical characterization of SC2-STs were performed through IFN-γ secretion and intracellular cytokine staining assays. These cells were expanded ex vivo after peptide stimulation, and isolated through MACS methodology on day 14. To determine the cytolytic capacity of isolated virus-specific T cells, a calcein-release assay with autologous PHA-blasts was performed. A CFSE-based proliferation assay was carried out to analyze the proliferative response of these cells.
Results: 9/12 donors (75%) presented a suitable number of $CD45^+CD3^+IFN\text{-}\gamma^+$ cells for their characterization, with a mean frequency of 0.019% ± 0.010% and a $CD4^+IFN\text{-}\gamma^+/CD8^+IFN\text{-}\gamma^+$ ratio of 1.76 ± 1.04. SC2-STs showed mostly T_{EM} phenotype in both $CD8^+$ and $CD4^+$ subsets (67.7% ± 20.0% and 55.4% ± 15.3% respectively), followed by T_{EMRA} in $CD8^+$ (20.4% ± 13.6%) and T_{CM} in $CD4^+$ (14.5% ± 9.8%). Granzyme B showed frequencies of 34.1% ± 21.3% in $CD8^+$ and 18.5% ± 20.1% in $CD4^+$, whereas most of the analyzed markers (Perforin, FasL, PD-1, CD38 and Ki-67) remained under 10%, with the exception of $CD8^+Perforin^+$ (18.0% ± 13.7%) and $CD4^+PD\text{-}1^+$ (29.4% ± 19.1%). The *ex vivo* expansion increased $CD45^+CD3^+IFN\text{-}\gamma^+$ to 3.92% ± 2.38%, with a median fold expansion of 294.4 (ranged 46.4-912). $CD8^+$ expansion showed great variability between donors, obtaining a heterogeneous population but an overall relative increase in the $CD4^+IFN\text{-}\gamma^+$ subpopulation ($CD4^+IFN\text{-}\gamma^+/CD8^+IFN\text{-}\gamma^+$ ratio of 2.25 ± 2.57). After MACS enrichment, the purity of the final product increased to 75.5% ± 11.0%, with 65.9% ± 22.7% yield and a ratio of 1.84 ± 2.17. Enriched cells showed *in vitro* SARS-CoV-2 specific cytotoxicity when co-cultured with SARS-CoV-2-pulsed PHA-blasts, and it depended on the relative proportion of $CD8^+IFN\text{-}\gamma^+$ cells and the target:effector ratio. Specific $CD3^+$ and $CD8^+$ demonstrated a significant reduction in CFSE median fluorescence intensity after antigen re-challenge with stimulated autologous feeders.
Conclusions: proven their functional capacity *in vitro* and their effector memory phenotype, IFN-γ-producing T-cells obtained from SARS-CoV-2 convalescent individuals could be used for the development of a T-cell therapy aimed to treat COVID-19-associated severe pneumonia.

PLE07-04

EXTENDING STABILITY OF WHOLE BLOOD FOR THE ANALYSIS OF T- AND B-CELL SUBSETS USING A LYSE-(FIX)-FREEZE METHOD

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Introduction: Flow cytometry is increasingly at the forefront of the analysis of pharmacodynamical changes upon treatment and immune monitoring in clinical trials. One of the challenges is the limited stability of surface and intracellular markers after blood collection, leading to logistical and scheduling challenges in clinical cytometry. Several strategies are available for sample stabilization, such as the use of blood collection tubes pre-filled with a fixative that can provide stability of up to weeks, and the isolation of PBMC, enabling long-term frozen storage. However, the fixation process can impact the ability of antibodies to bind their target, and PBMC isolation is not always feasible due to the limited availability of laboratory equipment or trained personnel. Therefore, we have investigated the possibility of freezing whole blood after lysis and/or fixation as another alternative to stabilize blood samples. Here, we present an easy and quick method for stabilizing markers expressed on T- and B-cell subsets for extended periods using minimal blood volume.
Methods: Whole blood was lysed with 10x excess of RBC lysis buffers and snap-frozen on dry ice and stored at -70°C for 2 months. As a control, whole blood in K3EDTA collection tubes was stored at 4°C. Samples were stained with a nine-color flow cytometry panel to distinguish T-cell subsets (e.g., T reg, CD4+ and CD8+ memory, and naïve T cells) and a five-color panel to analyze B-cell subsets (e.g. switched-memory, unswitched-memory, antibody-secreting B cells).
Results: Most of the T- and B-cell markers were stable at -70°C for up to 40 days in lyse-(fix)-frozen blood. Most of the subset percentages were stable as well, while clear disappearance of populations was observed in the whole blood stored at 4°C. T-cell and B-cell markers were stable for up to 1 week at 4 degrees.
Conclusions: Lyse-(fix)-freezing of whole blood is a straightforward method for stabilizing immune cell surface markers shortly after collection. This method is quick and requires minimal equipment and laboratory skills, enabling implementation at multiple clinical sites. We demonstrated stabilization of common T- and B-cell surface markers for up 40 days, therefore allowing batching of samples at the analytical laboratory. This can potentially reduce the costs of sample analysis and can improve data quality by reducing analytical variability.

PLE08-01

HOWARD SHAPIRO AND OTHER FALLEN SCIENCE HEROES - NO PEOPLE LIKE THE FLOW PEOPLE

J. Paul Robinson, Distinguished Professor of Cytometry, Purdue University

We often talk of the loss of our soldiers or our first responders, as fallen heroes. We honor them for their duty to our countries for giving us the freedom we enjoy. Scientists also have fallen heroes. They are those members of our community who led the charge of discovery, opened areas of science; educated us in the knowledge of our field and were the voices who pushed us to learn new things, and challenge old ideas.

Today, we celebrate the lives of our scientific heroes. We are so much better than we would have been without their teaching; their suggestions and sometimes their admonishments. As I learned in my first year at high school "*Science is the search for truth , by means of observation, reasoning, and experimentation*…" as taught to us by René Descartes almost 400 years ago.

For many of us in the cytometry community, the leaders of our field have always been there. They appear at conferences, they are in the audience, on the stage, in the bar. They are known by their scintillating presence at every meeting. Until they are not there. This past year has seen the loss of several of the most significant scientists in our field. Zbigniew Darzynkiewicz, James Watson, Michael Ormerod, Noel Warner, and Howard Shapiro. Let's consider these former members of our community and the impact they each had on us all.

POSTERS

Posters have been clustered according to the topic:

- Advances in Cytometry & Data analysis (ADV)
- Hematology-Oncology (HEM)
- Immunology (IMM)
- Other (OTH)

ADVANCES IN CYTOMETRY & DATA ANALYIS (ADV)

ADV-01

NEW MACHINE LEARNING-ASSISTED POPULATION IDENTIFICATION APPROACH FAITHFULLY REPLICATES MANUAL GATING OF HIGH-DIMENSIONAL FLOW CYTOMETRY DATA

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Introduction: Manual gating has long been identified as a major source of variability for flow cytometric analysis. Centralized data analysis as well as standardized gating strategies and stringent standard operating procedures have been suggested as a means to increase the reproducibility, but are time-consuming. Machine learning-assisted analysis of cytometry data has proven advantageous; however, there is a lack of methods that allow users to automate their own user-defined gating strategy for their specific marker panel in a hypothesis-driven setting. Here, we present an automatic gating approach that allows users to train a model on their manual gating results on a small number of samples without requiring coding skills.
Methods: Automatic gating models were trained using manual gating results from a subset of data from two different experiments, and the model tested on the remaining files. Model training included the automatic selection of the best performing models without intervention by the user. The datasets used for evaluation of the automatic gating approach included 96 replicates of the same PBMC sample stained using a T cell memory subsets panel, and analyzed by 4 independent operators, as well as the automated model and a 19-marker deep immunophenotyping panel on PBMCs from 20 healthy donors, comparing the analysis of one operator to a panel-specific automatic model.
Results: We show that our automatic gating models have reduced variability for populations defined by both scatter parameters and populations defined by fluorescence markers compared to manual gating and across several levels in a gating hierarchy. When comparing 10 different T cell memory populations across 4 operators to the automatic model for technical repeats of the same sample, the coefficients of variation (CVs) ranged between 2% and 139% for manual analysis (median: 6.75%), while the CVs for automatic analysis ranged between 1% and 19.4% (median: 2.7%). We demonstrate that automatic gating achieves a high correlation to manual gating. A total of 34 populations were manually gated on the deep immunophenotyping panel. A model trained on a subset of files achieved a median correlation across all populations of $R^2 = 0.99$ on previously unseen files.

Conclusions: By automating the identification of populations, assay reproducibility can be increased by removing inter-operator variability. We show that a supervised machine learning tool that supports user defined panels and gating strategies can reduce variability in flow cytometry data analysis.

ADV-02

EVALUATION OF THE PRESENCE OF NAIVE, ACTIVATED AND MEMORY T AND B CELLS IN RESPONSE TO SARS-COV-2 VACCINATION AND INFECTION

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Introduction: The adaptive immune system can provide efficient and lasting cellular and humoral immunity to viral infections, such as SARS-CoV-2, through T cell activation, differentiation, and protective memory. The aim of our study was to determine the presence of naive, activated and memory T cells in response to SARS-CoV-2 vaccination and infection as well as the detection of B cells (memory or naive) expressing IgG, IgM and IgA.
Methods: In order to investigate the presence of specific T and B cells against the Receptor Binding Domain (RBD) of the Spike protein of SARS-CoV-2, by Flow Cytometry, whole blood was collected and peripheral blood mononuclear cells (PBMCs) were isolated. For the T cells 4 different tetramers conjugated with PE and APC, specific for HLA-A2 (Human Leukocyte Antigen system) (BioLegend) were used and the markers CD45RA and CD27 were applied for the identification of naive T cells. For the B cells tetramers specific for RBD conjugated with PE and PE-Vio770 (Miltenyi Biotec) and markers for the detection of IgG, IgM, IgA expression were utilized as well as CD27 for memory cells classification. Samples from six individuals were collected prior and after the third dose of the vaccine. Eleven samples were collected after the third vaccination while eight samples were from individuals who were infected after their vaccination. All the participants were healthcare workers.
Results: No statistical significance was found in the percentages of specific T cells before and after vaccination, while statistical significant ($p<0,05$) reduction of naive ($CD45RA^+$) as well as double positive ($CD45RA^+$, $CD27^+$) T cells were observed in the samples that were collected after vaccination. In the sera of individuals who were vaccinated and infected naive T cells were less prevalent ($p<0,05$) than in sera of vaccinated individuals. No statistical significance was found for B cells expressing IgG and IgA and memory cells. Specific B cells for SARS-CoV-2 were increased after vaccination ($p<0,05$) while vaccinated with infection had significantly higher percent of IgM^+ specific B cells in comparison to the vaccinated without infection ($p<0,05$).
Conclusions: After vaccination specific for SARS-CoV-2 naive T cells decreased, that is indicative of T cell activation. Infection after vaccination also demonstrates higher T cell activation. Specific B cells for SARS-CoV-2, as expected, were increased after vaccination, while higher percentages of IgM^+ B cells were found in vaccinated that were also infected.

ADV-03

IMPACT OF RED BLOOD CELL LYSING ON RARE EVENT ANALYSIS

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Introduction: Rare cells are low-abundance events found at a frequency equal or lower than 0.01%. An important consideration for rare cell analysis by flow cytometry (FC) is sample preparation. Lysing erythrocytes is commonly performed when processing hematic specimens for FC immunophenotyping. However, erythrolytic solutions can have important consequences on cell immunophenotyping, such as a cell loss, changes in the scatter properties, and shifts in fluorescence intensity. Alternatively, sample preparation protocols avoiding lysing steps are simpler and can avoid these consequences by minimizing sample manipulation. In this study, we have used minimal sample perturbation techniques previously described (Rico et al., 2021) to detect dendritic cells (DCs) and to evaluate measurable residual disease (MRD) in comparison with conventional methods.
Methods: EDTA-anticoagulated blood samples were obtained from patients and healthy donors in Hospital Germans Trias i Pujol (Badalona, Spain), after receiving informed consent. For minimal sample perturbation assays, samples were stained with Vybrant™ DyeCycle™ Violet Stain (DCV) and incubated for 20 min at 37 °C protected from light. DCV is a cell-permeant low-cytotoxic nucleic acid stain that allows excluding non-nucleated cells and cell debris. DCs and MRD were evaluated by FC immunophenotyping with the appropriate monoclonal antibodies. Absolute numbers and frequency of detected rare cells were compared with rare cells detected when using conventional methods for sample preparation, involving the use of erythrolytic solutions.
Results: When using lysing solutions, a reduction of the absolute number of DCs was observed (n=20). When comparing with unlysed samples, using CyLyse™, OptiLyse C and Pharm Lyse™, decreased the absolute number of conventional DCs was significantly. (p-value<0.0001, =0.005 and =0.0001; respectively). The absolute number of plasmacytoid DCs was significantly reduced when using CyLyse™ (p-value>0.0001) and Pharm Lyse™ (p-value<0.0001). When evaluating MRD, all lysed samples (n=3) showed a reduction in leukocytes and leukemic cells (LCs). When avoiding lysing solutions, 59.75 leukocytes/µl and 0.564 LCs/µL were detected in one representative sample, whereas the lysing method gave 31.69 leukocytes/µl and 0.456 LCs/µL.
Conclusions: All these results may have implications for rare cell detection, indicating that a reduction of sample manipulation is essential for accurate measurements. The use of erythrolytic solutions may lead to MRD overestimation if lysing-resistant cells exist.

ADV-04

METAFLOW - AN INNOVATIVE TOPOLOGICAL ANALYSIS PLATFORM FOR HIGH-DIMENSIONAL FLOW CYTOMETRY DATA

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Introduction: Continuous increase in number of markers of flow cytometry based assays induce development of numerous analytical tools capable of dimensionality reduction and/or clustering essential to the exploration and interpretation of the multidimensional data. Available to date solutions are powerful and designed to represent the original data structure, but in many instances they fail to deliver as they rely only on a number of subjective variables that impact the results and ultimately the interpretation. In this work we present a cloud based platform that maintain the original data structure as dictated by the values and signal resolution generated at acquisition. Analysing relatively simple panel with a near automated output shows real world relevance to clinical samples.
Methods: The Metaflow platform was used to analyse a well-defined 8 marker fluorescent panel acquired on a conventional fluorescence flow cytometer. Data was also analysed via "ground truth" manual gating as well as FLOWSOM clustering with UMAP visualisation using FCS express software (De Novo) for comparison.
Unlike the aforementioned approaches, one can go directly with the native data set after compensation. METAflow automatically performs scaling transformation as adapted to the method of data acquisition (conventional, spectral, mass). This keeps the entire structure of the data and clusters by taking into account all available parameters. Additionally, there are inbuilt modules for signal instability removal and identification of debris and doublets which were vetted against manual gating approaches to the same end.
Results:
Metaflow based analysis was shown to provide high accuracy results and allows for preservation of the original data structure. Debris was readily clustered and, automated labelling of debris and doublets was demonstrated to significantly shorten analysis time per sample. The seamless, integrated design of the solution made it insensitive to prior gating, increasing robustness and reproducibility of the end-point analyses.
Conclusions:
Metaflow offers seamless workflow relying on density-based algorithm that maintains the original data structure akin to auto-gating. This can provide detailed insight into data granularity while operating in multiple dimensions. The clustering algorithm further optimizes achieving robust and rapid data presentation by the cleaning of both doublets and debris, expediting computational time to results. Metaflow can be a gateway for valid and reproducible discovery in data sets, accessible and sharable to any user who has no prior knowledge in the multistep process of current overly-complex automated strategies and lays a promising basis to develop a digital clinical cytometry research and diagnostic approaches.

ADV-05

PRODUCTION AND CHARACTERIZATION OF EXTRACELLULAR VESICLE REFERENCE SAMPLES FOR RIGOROUS AND REPRODUCIBLE EV ASSAYS

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Introduction: Extracellular vesicles (EVs) are small lipid nanoparticles, released from all cells. EVs are of interest in a variety of therapeutic, diagnostic, and biological contexts. Their clinical utility, however, is hampered by challenges inherent to reliably measuring EVs which are approximately 10^5-fold smaller than cells by surface area. Incorporation of well-characterized standards are a tenet of rigorous, reproducible, and interpretable assays which are especially important for challenging measurements.

Recommendations for standards and controls for EV characterization are described in recently published guidelines such as MIFlowCyt-EV. Assays such as vesicle flow cytometry (vFC) incorporate these standards to achieve practical and reliable measurement of EVs. vFC includes several standards and controls to ensure EV specificity, single EV resolution, and interpretability of positive and negative EV surface cargo measurements. Herein we describe the production of EV reference samples from human platelets and cell lines as positive controls for EV surface cargo measurement by vFC.
Methods: Platelets purchased from the San Diego Blood Bank were washed and treated with calcium ionophore (A23187) to induce vesiculation. The resulting vesicles were enriched by low-speed centrifugation. Human cell lines or HEK293T cells engineered to express specific markers of interest were cultured in either flasks or hollow fiber bioreactors as appropriate. Cell culture supernatants were harvested and EVs were enriched by ultrafiltration as required. Measurement of common surface markers such as the tetraspanins CD9, CD63, and CD81 was then performed on calibrated flow cytometers as described in the vFC protocols.
Results: Extracellular vesicles derived from human platelets, U87 cells and HEK293T cells expressed high concentrations of and were identified as appropriate positive controls for the tetraspanins CD9, CD63, and CD81 respectively.
Discussion: Future work to identify or engineer additional reference materials for other markers of interest will facilitate rigorous and reproducible measurement of additional surface cargo important to therapeutic, diagnostic, and basic research applications.

ADV-06

MIFlowCyt-EV REPORTING OF SINGLE VESICLE FLOW CYTOMETRY METHODS AND RESULTS

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Introduction: Rigor and reproducibility are major issues for science in general, and the EV field in particular. It is increasingly appreciated that the key to data interpretation lies in the details of the methods used to produce the data. Single vesicle analysis using flow cytometry is a case in point, with literally hundreds of different ways to prepare and measure a sample using a flow cytometer, and the details of those methods and validation of their performance are required for understanding the data. The recent MIFlowCyt-EV guidelines address this need by providing a checklist of issues that must be addressed to enable data interpretation and sharing. Here we illustrate an assay and reporting methods that enable characterization of EVs in a MIFlowCyt-compliant manner.
Methods: We measured individual EVs in culture media and plasma using a commercial flow cytometer and assay kit. Preanalytical steps included pelleting of cells, dilution, and/or concentration by ultrafiltration. The flow cytometers were qualified and calibrated using calibration beads to enable data reporting in standardized units. EV concentration, size, and surface cargo was measured by single vesicle flow cytometry (vFC) which incorporates validated reagents, essential controls, and standardized protocols and data analysis.
Results: We report size, concentration, and surface cargo (CD9, CD63, CD81, and PLAP) molecule number per EV in standard units on EVs from blood and cell culture. Calculation of these parameters is described within the context of the sample preparation and assay protocols. Example calibration and control data that might be included in supplementary methods as suggested by the MIFlowCyt-EV ISEV position paper is reported. Data analysis protocols are illustrated with examples.

Discussion: Single EV analysis using flow cytometry offers great potential for understanding the diverse origins and functions of EVs, but only if performed in a way that allows assay specificity and sensitivity to be transparently documented. The MIFlowCyt-EV guidelines as addressed by vFC provide a practical approach to measurement and reporting of the essential characteristics of EVs in the context of essential experimental details, calibrations, and controls. Many of the concepts and procedures developed for EV FC are extensible to other single EV counting, sizing, and cargo analysis methods.

ADV-07

QUANTITATIVE ANALYSIS OF MOLECULAR CARGO TRANSFER FROM TUMOR CELLS TO EVs

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Introduction: EVs carry molecular cargo from their cell of origin, and are attractive as potential liquid biopsy targets, but the mechanisms of cargo selection and loading into EVs are not well understood. We used quantitative single cell and vesicle flow cytometry to measure membrane protein expression on cells and EVs with the aim of better understanding how tumor cell cargo is released via EVs.
Methods: PC3 prostate cancer cells were cultured, the media collected, and EVs concentrated using ultrafiltration (100K MWCO). Cell surface markers were measured by flow cytometry (FC). EV concentration, size, and cargo were measured by single vesicle flow cytometry (vFC). Instruments were calibrated and intensity reported in units of antibodies per cell or EV.
Results: PC3 cells express surface markers at high (>250K median copies/cell: CD71, CD29, CD44, CD54), medium (50K-250K copies: CD9, CD63, CD49f) and low (<50K copies: CD81, EPCAM, EGFR, STEAP-1) abundances. Cell permeabilization reduced staining for CD9 and CD81 (due to disruption of the plasma membrane), and increased staining for CD63 and STEAP1 (due to accessibility of internal antigen). EVs expressed detectable (>~10 PE MESF) CD9, CD63, CD81 and CD29, with a fraction (~50%) also staining with AnnV. Expression was proportional to EV surface area, with surface densities ranging from a background of ~10 molecules/um^2 to >1000 molecules/um^2 for high abundance targets. Several high abundance markers (CD71, CD44, CD54) were not detectable on EVs, suggesting differential packaging of cell surface cargo into released EVs. CD63 was expressed at low abundance overall, but a subset of smaller EVs (<100 nm) expressed CD63 at high surface density (~1000 um2).
Conclusions: We find that the abundance and surface density of cargo on vesicles can be higher or lower than on the cell of origin. Some abundant cell surface molecules (CD71, ICAM, CD44) were undetectable on EVs, while others (CD9, CD81, CD29) were present at surface density similar to cells. CD63 was present at high density on smaller EVs, consistent with enrichment of CD63 on small exosomes formed inside the cell.

HEM-01

CHANGES IN MONOCYTES FOLLOWING TREATMENT OF AML PATIENT WITH COMBINATION OF VIDAZA AND VENETOCLAX

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Introduction: A combination of Vidaza and Venetoclax showed promising results in older Acute Myeloid Leukemia (AML) patients with pre-existing medical conditions. However, half of the patients relapse, probably due to Venetoclax resistance.Venetoclax resistance was found to correlate with high monocyte count, and myelomonocytic-monoblastic leukemia (M4/M5). It was suggested that the increased monocytic population in AML correlates with combined Vidaza and Venetoclax resistance. However, to the best of our knowledge, phenotypic changes, have not been described. Herein we show representative AML patient samples demonstrating several myeloid-monocytic phenotypic changes during and after combined Vidaza and Venetoclax treatment**.** Our aim is to evaluate phenotypic changes in AML patient treated with this combination.
Method: Bone Marrow (BM) populations were analyzed on a Navios Flow Cytometer. Samples were evaluated for phenotypical changes using standard flow-cytometric procedures and recommended AML screening antibodies.
Results: The main changes we observed in AML patients after combined Vidaza and Venetoclax treatment were: Increase in the size of the monocyte gate, Different monocyte subpopulations distributed within the monocyte gate (Classical / Intermediate / non-classical monocytes) , phenotypical aberrations including (decrease in HLA-DR expression, increase in CD56) and changes in CD14 Expression.
Conclusions: Following Vidaza and Venetoclax combined treatment in AML patients, we show an increase in the size of the monocyte gate. It has been suggested that the increased monocytic population in AML correlates with resistance to combined Vidaza and Venetoclax treatment. Therefore, it is tempting to speculate that these induced monocytic populations in our patients may be the cause of resistance to the therapy. Phenotypic changes such as HLA-DR reduction and aberrant monocytes subpopulations which may affect the immunological response were also demonstrated. The observed increase in CD56 may indicate a poor prognosis. All of these above described changes may affect AML cellular response to therapy.

HEM-02

EXPRESSION PATTERN OF CD244, A NOVEL SLAM PROTEIN AND ITS CLINICAL UTILITY IN THE DIAGNOSIS OF ACUTE leukemia

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Introduction: CD244, a member of signalling lymphocyte-activation molecule (SLAM), is expressed on NK-cells, subpopulation of T-cells, monocytes, and basophils. CD244 is important in maintenance of stemness of leukemia-initiating cells. However, there is lack of studies evaluating its expression in acute leukemias (AL). With the advent of immunotherapy, we aimed to assess expression of CD244 in AL patients and evaluate its role as therapeutic target.
Methods: Flowcytometric immunophenotyping was performed to study CD244 expression(PECy7, C1.7) on normal cells and leukemic-blasts using 10-13 color panel. Cells were acquired on 3-laser 13-colour Cytoflex (Beckman Coulter, USA). The study included 54 B-ALL, 49 T-ALL, 92 AML, 20 MDS, and 7 uninvolved bone marrow (BM) samples submitted for staging (controls). Post-acquisition analysis was performed using Kaluza-v2.1 with pre-defined template. CD244-positivity was defined with cut-off of ≥10% CD244-positive leukemic-blasts, in accordance with AIEOP-BFM Guidelines. Statistical evaluations were performed using MedCalc-v14.8.1.
Results: CD244 was positive in 5/54 (9.26%) B-ALL, 24/49 (48.98%) T-ALL, 89/92 (96.74%) AML, and 20/20 (100%) MDS cases. Evaluation of controls showed CD244 expression on monocytes, NK and T-cells. The median (range) CD244 nMFI in monocytes was 6.46 (1.08–8.60), NK-cells was 5.63 (0.11–9.31), and T-cells 2.69 (0.30–7.78). Median (range) CD244 nMFI in leukemic-blasts of AML was 8.96 (0.23–48.42), T-ALL was 0.41 (0.00–9.96), and B-ALL 0.03 (0.00–5.74). The median (range) CD244 nMFI in MDS-blasts was 8.33 (1.35–9.71). CD244 expression was assessed in granulocytes and monocytes from AML and MDS. The median (range) CD244 nMFI in granulocytes and monocytes from AML were 3 (0.00–9.46) and 4 (0.23–10.00) respectively, and 2 (0.27–6.24) and 10 (3.69–10.55) from MDS. CD244 expression distinguished AML-blasts from granulocytes ($p<0.0001$) and monocytes ($p<0.0001$), and MDS-blasts from granulocytes ($p<0.0001$) and monocytes ($p = 0.0002$).
In T-ALL, CD244 expression on NK-cells distinguished CD244-negative T-blasts from NK-cells ($p<0.0001$). The median (range) CD244 nMFI in NK-cells and T-blasts was 8.23 (0.04–9.99) and 0.41 (0.00–9.96) respectively.
Conclusion: Current study is the first to evaluate CD244 expression in AL. CD244 is widely expressed by AML-blasts and myeloid-blasts. In T-ALL, it helps identify NK-cells from T-blasts. Our data highlights role of CD244 as potential marker for MRD-monitoring in AML where blasts can be distinguished from granulocytes/monocytes, and T-ALL where T-blasts can be distinguished from NK-cells. The expression of CD244 on AML-blasts and myeloid-blasts indicates its role as a potential target for immunotherapy, however this will require validation. Nonetheless, our study demonstrates expression pattern of CD244 across the leukemic-blasts in AL.

HEM-03

HYPOXIA SIGNALLING PATHWAY AND THE ENZYMES INVOLVED IN DNA DEMETHYLATION IN ACUTE MYELOID AND LYMPHOBLASTIC LEUKEMIA PATIENTS

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Introduction: The aim of this study was to compare intracellular expression of enzymatic proteins: TET (ten-eleven translocation), TDG (thymine DNA glycosylase) involved in epigenetic processes (DNA demethylation), as well as, HIF-1AN and HIF-1alpha (hypoxia signalling pathway) in populations of peripheral blood nuclear cells in adults patients with acute myeloid leukemia (AML) and children with acute lymphoblastic leukemia (ALL).

Methods: Peripheral blood samples were collected into tubes containing EDTA and the cellular surface antigen-stabilizing agent. The blood were stained by anti-human antibodies (CD56/CD16-PE, CD19-APC-H7, CD45-BV421, CD3-V500, CD34-PE-Cy7) for identification of blast cells and peripheral blood nuclear cell populations (lymphocytes, monocytes, granulocytes). Next, the cells were fixed, permeabilized and co-stained by indirect method with anti-human primary antibodies (TET-1, TET-2, TDG, HIF-1AN and HIF-1alpha) followed by compatible secondary antibodies conjugated with fluorescent dyes (donkey anti-rabbit Alexa Fluor-488 or rabbit anti-goat Alexa Fluor-647). The expression of analyzed proteins was calculated as fluorescence intensity fold change over negative control.
Results: In all investigated cell populations, patients with AML showed significantly higher expression of TET-1 compared to patients with ALL. The highest expression of TET-1 was observed in the population of blasts and monocytes. At the same time, decreased expression of TET-2 was demonstrated in the population of monocytes of AML patients. Moreover, an increased expression of TDG was found in the blast cells and monocytes of AML patients vs. ALL. The assessment of intracellular expression of hypoxia signalling pathway enzymes showed a significantly lower expression of HIF-1AN in the granulocyte population of AML patients. There were no differences in the expression of HIF-1alpha in all groups and populations of cells.
Conclusions: High expression of TET-1 (PCR-study) has already been described in patients with AML and it has been shown to be related to poor survival of patients. In our study, increased expression of TET-1 was observed in patients with AML in all investigated cell populations vs ALL. Interestingly, the highest expression of TET-1 is seen in the population of AML blast cells and monocytes. A similar tendency is observed in TDG expression (the highest values in the population of blasts and monocytes in AML patients). Simultaneously the results of HIF-1AN and HIF-1alpha expression indicate a probable lack of correlation between the processes of hypoxia and the expression of TET and TDG enzymes. This work was supported by the University Center of Excellence Towards Personalized Medicine.

HEM-04

Performance of BD oneflow™ PCST and BD oneflow™ PCD Dried Reagents on BD facslyric™ and BD facscanto™ II Systems

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Introduction: This multisite method comparison study was conducted at four European clinical laboratories using de-identified remnant bone marrow (BM) specimens from subjects with presumptive or having plasma cell diseases. The objective was to demonstrate equivalent performance of the BD OneFlow™ PCST (PCST) and BD OneFlow™ PCD (PCD) Dried Reagent Tubes on the 10-color BD FACSLyric™ and BD FACSCanto™ II Flow Cytometry Systems.

Methods: Ethics review was managed according to site procedures and country guidelines. BM samples were prepared following to reagents instructions for use and acquired on the BD FACSCanto™ II and BD FACSLyric™ Systems. A site expert determined normal or abnormal plasma cell phenotypes. Results were analyzed using 2X2 contingency table to calculate overall percent agreement (OPA), positive percent agreement (PPA) and negative percent agreement (NPA) with 95% confidence interval (95% CI) between the two systems.
Results: BMs were collected in EDTA (K2=45/K3=31) or heparin (lithium=3/ sodium=1). Compliant results from PCST (80) and PCD (78) were included in the analysis. For PCST, 42 and 38 specimens were classified having normal or abnormal plasma cell phenotypes; for PCD, 42 and 36 specimens had normal or abnormal cell phenotypes. Designation of normal and abnormal plasma cell phenotype with PCST and PCD gave 100% OPA, PPA and NPA values with 95% CI (92.22% and 96.32%) between flow cytometry systems.
Conclusion: Agreement results indicate equivalent performance of PCST and PCD reagents on the BD FACSLyric™ and BD FACSCanto™ II Systems.

HEM-05

PHENOGRAPH AND tSNE IDENTIFICATION OF MATURE T-CELL NEOPLASMS AND MONOCLONAL T-CELL POPULATIONS OF UNDETERMINED SIGNIFICANCE

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Introduction: The diagnosis of T-cell neoplasms is often complicated due to a wide range of phenotypic abnormalities, many overlapping features with reactive T-cells and limitations of available T-cell clonality assays. The aim of this study was to evaluate if Phenograph clustering algorithm and dimensionality-reduction algorithm tSNE could help in identifying monoclonal T-cell populations using antibody specific for T-cell receptor β-chain constant region 1 (TRBC1).
Methods: Fifty-five samples from patients with a definitive diagnosis of mature T-cell neoplasm (peripheral T-cell lymphoma, not otherwise specified (32), angioimmunoblastic T-cell lymphoma (11), Sezary syndrome (7), mycosis fungoides (4) and T-cell large granular lymphocytic leukemia (1)) and 70 samples from patients with no T-cell neoplasm (reactive lymphocytic proliferation (32), B-cell lymphoma (28), Hodgkin lymphoma (7) and unexplained leukocytosis (3)) were included in this study. Specimen types included 65 lymph node biopsies, 30 bone marrows, 23 peripheral bloods and 7 pleural effusions. All samples were evaluated by 10-color flow cytometry antibody panel TRBC1 FITC/CD2 PE/CD5 PerCp-Cy5,5/TCRγδ PE-Cy7/CD7 APC/CD19 APC-R700/CD8 APC-Cy7/CD3 V450/CD45 V500/CD4 BV605 and T-cell gene rearrangement studies were performed using BIOMED-2 clonality assay (Invivoscribe). Manual gating, Phenograph and tSNE analysis were performed. Distinct CD4 and CD8 T-cell populations (manual gating) and algorithm generated clusters were evaluated for TRBC1 expression. T-cells expressing less than 15% or more than 85% of TRBC1 were classified as monoclonal.

This study was approved by The National Medical Ethics Committee of the Republic of Slovenia (0120-165/2020/4).
Results: Of 55 samples of T-cell lymphoma, Phenograph/tSNE and manual gating analysis detected neoplastic monoclonal populations using TRBC1 antibody in 53 (96,4%) and 52 (94,5%) samples, respectively. PCR clonality assay detected monoclonal cells in 49 samples (89,1%). Six samples (10,9%) showed presence of monoclonal products that didn't meet all criteria for full monoclonality. In 2 samples (3,6%) no monoclonal neoplastic T-cell populations were detected with any method. Of 70 non-T-cell lymphoma samples Phenograph/tSNE analysis identified 41 (58,6%) samples with monoclonal T-cell population. PCR clonality assay identified 43 (61,4%) samples and manual gating identified 35 (50%) samples with monoclonal T-cell populations.
Conclusions: Phenograph clustering algorithm and dimensionality-reduction algorithm tSNE used with TRBC1 antibody show great promise as an automated method for immunophenotypic identification of T-cell clonality in mature T-cell neoplasms.

HEM-06

A CASE OF LARGE GRANULAR LYMPHOCYTE LEUKEMIA WITH AN UNUSUAL IMMUNOPHENOTYPE

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Introduction: Large granular lymphocyte leukemia (LGLL) is a rare type of chronic mature lymphoproliferative disorder of the T- or natural killer (NK) lineage constituting 2-5 % of chronic lymphoproliferative disorders in Europe[1,2]. It is often indolent with few clinical symptoms, and typically presents with persistent neutropenia and anemia[3]. About 40-60 % of the patients have immunological abnormalities[4]. The diagnosis is set by identifying an expanded clonal T-or NK-cell LGL population through examination of blood smears, flow cytometry and T cell receptor (TCR) gene rearrangement studies, usually by polymerase chain reaction (PCR) analyses[1]. T-LGLL accounts for 85 % of the cases and most of these express αβ TCR. We present a case of T-LGLL with an unusual immunophenotype.
Methods: A 53 year old woman was referred to a specialist in hematology due to anemia and neutropenia for the last 6-12 months. She had a 28 year history of Sjögrens's syndrome with autoantibodies; anti-Ro/SSA, anti-La/SSB and anti-dsDNA. She was clinically asymptomatic.
Results: There was no lymphadenopathy or other findings by physical examination, though an abdominal computed tomography (CT) examination demonstrated splenomegalia. Laboratory results showed hemolytic anemia (Hbg: 9.6 g/dl, LD 288 U/L, haptoglobin <0.10 g/L, reticulocytes 0.14 x 10^{12}/L, bilirubin: 13 µmol/L, direct antiglobuline test: negative), lymphocytosis (lymph: 5.2 x 10^9/L), and neutropenia (neutrophils: 0.5 x 10^9/L). Examination of peripheral blood smear showed lymphocytosis, whereas 60 % of the cells were characterized morphologically as LGL-cells. Immunophenotyping of blood and bone marrow identified a population of 50-60 % of viable white cells with immunophenotype: CD45+, 45RA+, 45RO-, 2+, 3+, 4-, 5-, 7 weak, 8+, 16 heterogenous, 19-, 20-, 26-, 27-, 28-, 56-, 57-, 94-, cyPerforin-, cyGranzymeB+, TCR γδ+. Histopathological analysis of bone marrow biopsy demonstrated diffuse lymphocytic infiltration of CD8+ T cells. PCR confirmed the diagnosis of Tγδ LGLL.
Conclusion: We present a case of Tγδ LGLL, a variant that represents only 5 % of all T-LGLLs and is relatively scarcely described in literature[5,6,7]. LGLL is an uncommon condition. As it often presents with few clinical symptoms, and many of the patients already have autoimmune disorders, it may be diagnostic challenging. Awareness of the disease and the different subtypes is therefore important.

HEM-07

Daratumumab effect on Minimal Residual Disease of Multiple Myeloma patients in Very Good Partial Response by using Next Generation Flow

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Introduction: Daratumumab (Dara) is an effective anti-CD38 monoclonal antibody for the treatment of Myeloma patients. High-sensitive techniques permit to monitor Minimal Residual Disease (MRD) of MM patients and, in this context, Next Generation Flow (NGF) is a high-standardized assay that permits, in a rapid way, to define MRD negativity. We want to confirm that NGF is a powerful tool for detection of MRD of MM patients in Very Good Partial Response (VGPR), during consolidation therapy with Dara, and to determine Datumumab efficacy, and its role in prolonging Overall Survival (OS) and Progression Free Survival (PFS) of MM patients.
Methods: Starting from December 2018, a pilot study has been set up in Siena. MM patients in VGPR, MRD positive by NGF, were enrolled and MRD evaluation was assessed at 2 months of treatment, and every 6 months up to 2 years. Patients who are negative at 6 mos will stop Dara treatment, while patients still MRD positive will continue Dara up to 2 years.
Results: 50 MM patients have been enrolled up until now, reaching the goal of the study. Of 46/50 (92%) MM patients who reached the first endpoint of 6 mos of Dara treatment, 14/50 (28%) achieved MRD negativity, of which 8/14 (57%) were “early responders” and could obtain MRD negative status already at 2 mos. 37/50 (74%), 26/50 (52%) and 21/50 (42%) patients have been evaluated at the timepoints of 12, 18 and 24 mos respectively, with 3/8 (37%) of the “early responders” patients, negative since 2 mos, persisting in MRD negative status. The high heterogeneity of response obtained needs to be correlated to type of treatment received before starting Dara, as the study confirms that undergoing at least one Autologous Stem Cell Transplantation (ASCT) can help reducing the pathogenicity of MM and achieving a deeper response; cytogenetic risk correlations, instead, need a larger cohort of patients. Lastly, by looking at the percentage of clonal vs normal PCs in patients who maintain a MRD positive status, we observed a decrease of pathologic MM PCs during Dara treatment, and a tendency to “stabilize” with a low percentage at longer timepoints from Dara treatment.
Conclusions: Daratumumab is effective as consolidation therapy in MM patients in VGPR, as it induces MRD negativity and could help “checkmate” pathologic plasma cells, that may persist in MRD positive patients but with a very low tumor burden.

HEM-08

CLINICAL UTILITY OF CD177 IN THE DIAGNOSIS OF MYELODYSPLASTIC SYNDROME

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Introduction: CD177, also known as the NB1 or HBA-2 antigen, is a glycosylphosphatidylinositol (GPI)-linked cell surface antigen expressed exclusively on neutrophils. Neutrophils show a bimodal expression pattern of CD177. Both the fraction and expression level of CD177 increase during neutrophil maturation with approximately 45% to 65% of mature segmented neutrophils expressing the protein. Multicolour flow cytometry (MFC) is a rapid and useful tool in the diagnosis of myelodysplastic syndrome (MDS). Therefore, we studied the downregulation of CD177 in CD11b strong (+) and CD16 strong (+) granulocytes (neutrophils) as an additional parameter for the MFC diagnosis of MDS.
Methods: We studied the expression of CD177 (APC, REA258) on mature neutrophils (CD16++ & CD11b++) in bone marrow samples of MDS (n=30) and non-myeloid neoplasm(n=50) (including staging marrows) cases using a 10-13 color panel. Cells were acquired on Cytoflex (Beckman Coulter) and data was analyzed using Kaluza software v2.1.
Result: CD177 expression was studied in a total of eighty cases (fifty non- myeloid & thirty MDS). Median (range) of CD177(+) neutrophils in non-myeloid neoplasms and in MDS cases were 79.18 (23.48%-99.6%) and 22.84(1.6%-96.6%) respectively. We observed pathogenic loss of CD177 and determined a cut-off of <36% CD177(+) neutrophils in MDS using the ROC method. Loss of CD177(<36.0%) was observed in 63.3% (19/30) cases with a median(range) of 22.4(1.6-35.2). The specificity and sensitivity of CD177 loss in the diagnosis of MDS was respectively 92% and 66.67.0% (p=0.0001). In 21/30 cases, granulocytes showed abnormal maturation-patterns with respect to CD13/CD16/CD11b which also showed loss of CD177 in 17/21 (81%) cases. No abnormal maturation patterns were observed in the remaining 09/30 MDS cases, but the loss of CD177 as a sole immunophenotypic abnormality in 02/9 (22.2%) cases were seen. Cytogenetics studies could be performed in 26/30 MDS cases and 13/26 cases show cytogenetic abnormality (either monosomy 7q, deletion 5q, trisomy 8, or TP53 deletion). Loss of CD177 with a cutoff of <22.5% was associated with trisomy 8 (p=0.04). However, more studies are needed to confirm this finding.
Conclusion: Downregulation/loss of CD177 in mature granulocytes (<36.0%) is a distinct feature of dysgranulopoiesis and valuable addition to immunophenotypic abnormalities of MDS. Hence, it can be incorporated into MFC diagnosis and scoring systems for MDS.

HEM-09

GATA-3 IS A HIGHLY SENSITIVE AND SPECIFIC MARKER FOR THE T-CELL LINEAGE ASSIGNMENT IN THE IMMUNOPHENOTYPIC CLASSIFICATION OF ACUTE LEUKEMIA

Niharika DasGupta [1], Prashant Tembhare [1], Anu Singh [1], Juee Ranade [1], Sitaram Ghogale [1], Nilesh Deshpande [1], Yajamanan Badrinath [1], Karishma Girase [1], Harshini Sriram [1], Sweta Rajpal [1], Gaurav Chatterjee [1], Nikhil Patkar [1], Sumeet Gujral [2], Papagudi Ganesan Subramanian [1]

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Introduction: GATA-binding protein3(GATA3) is a transcription factor known to play a role in early T-cell differentiation. Current T-lineage definition in acute leukemia(AL) requires expression of surface or cytoplasmic CD3 up to the level of mature T-lymphocytes. However, sometimes, in routine practice, cytoplasmic CD3 shows dim to negative expression, and T-cell-associated markers including CD7, CD5, CD4 and CD2 are frequently expressed in other leukemias and are not T-lineage-specific. In cases of mixed-phenotypic AL, it becomes particularly challenging to confirm the diagnosis. Hence, there is a requirement for an additional marker that can help in the identification of T-lineage. In the present study, we standardized GATA3 expression by multicolor flow cytometry(MFC) and evaluated its utility as a T-lineage marker in the lineage assignment of AL.

Methods: Anti-GATA3(PECF-594, clone-L50-823) antibody staining was standardized and studied in the leukemic blasts using MFC. Intracellular staining was assessed using four different permeabilization reagents: FACS Lyse(BD Biosciences), Fix-&-Perm(Invitrogen), Foxp3-fixation-kit(eBiosciences) and True-nuclear transcription-factor staining buffer-set (Biolegend). Expression level of GATA3 was determined as normalized mean fluorescent intensity(nMFI) against internal negative control.
Results: Of the four reagents studied, FACS Lyse (BD Biosciences) was found to be the best permeabilization reagent for GATA3 expression. We studied 138 T-ALL, 32 AML, 18 MPAL, and 20 B-ALL patients. Mature B-cells and T-cells were taken as negative and positive controls with a median(range) of nMFI of 0.52(0.0 -8.95) and 1.32(0.0 - 6.73), respectively. Taking a cut-off of 2% positivity defined using ROC analysis, 138/138(100%) T-ALL and 13/18(72.2%) MPAL(15 T/myeloid and 3 B/T lymphoid), were positive and 20/20(100%) of B-ALL cases were negative for GATA3 expression. We also observed that 4/32(12.5%) cases of AML were also positive for GATA3 and had CD7 co-expression. Median(range) of nMFI of GATA3 in T-ALL, AML, MPAL, NK ALL, and B-ALL patients were 3.50(0.0-9.47), 0.17(0.00-6.32), 1.56(0.00-9.38), 5.93(0.00-5.93), and 0.00(0.00-0.10) respectively. For safer analysis, we adapted a cut-off of 5%(instead of 2%) to define GATA3 positivity. The sensitivity and specificity of GATA3-expression for T-cell lineage using a cut-off of 5% positivity were 96.4 and 100% respectively.
Conclusion: We first-time standardized flow cytometric assessment of GATA3 expression in the clinical setting. FACS Lyse(BD-Biosciences) was the best permeabilization reagent. We conclude that flow cytometric GATA-3 is a sensitive and specific marker for the diagnosis of acute leukemias with T-cell differentiation and its expression can provide additional evidence in the assignment of T-lineage in difficult cases of AL with weak cytoplasmic-CD3 expression.

HEM-10

EXPRESSION PATTERN OF CD179α IN B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (B-ALL): A POTENTIAL UTILITY FOR LINEAGE ASSIGNMENT AND B-ALL MRD MONITORING

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Introduction: CD179α/β (VpreB/λ5) is a surrogate light chain, present on precursor B-cells which is replaced by conventional κ & λ light chain consisting of CD79α/β (Igα/Igβ) on mature B-cells. A relatively restricted repertoire of markers is shared by most B-ALL cases. Moreover, assignment of B-lineage by flowcytometric immunophenotyping (FCI) can be challenging in a few cases with weak-CD19 expression or only two B-cell markers (CD10/CD22/CD79a) are positive as per WHO criteria. This study aims to assess the expression of CD179α to evaluate its potential utility for lineage assignment, MRD monitoring, and targeted therapy in B-ALL.
Methods: CD179α (BV421, APC; clone-HSL96) antibody staining was standardized and its expression was studied in diagnostic samples of BALL using 10-color panel. The staining protocol for CD179 expression was standardized. MFC was performed on 13-colour DxFlex flow cytometer (Beckman Coulter) and data was analyzed using Kaluza-v2.1-software. Expression-level of CD179a was studied using normalized mean fluorescent intensity (nMFI) and expression-pattern (heterogenous/homogeneous) using CV of immunofluorescence (CVIF). Its expression was also studied in hematogones and mature B-cells. Normal T-cells within samples were taken as a negative control.

Results: Of two staining protocols, cytoplasmic staining using Fix-&-Perm (Invitrogen) permeabilization reagent was found to be best for cytoCD179a expression. CD179α was studied in 55 B-ALL patients with a median (range) positive percentage in B-ALL cells of 73.46% (6.11%-98.65%). Using ROC method, a cut-off of >5% was used to define CD179α positive expression. Median (range) of nMFI of CD179α in B-ALL blasts, hematogones, mature B-cells and T-cells were 5.65(2.15 – 19.31), 23.31(9.26 -28.29), 4(0.41 – 14.1) and 0.49(0.12 – 2.38) respectively. Median (SD) of CVIF of CD179a in hematogones was 89.67(25.31) very low compared to that of B-ALL i.e. 159.86 (199.88)]. Thus, CD179α expression was found to be aberrantly downregulated and heterogeneous in B-ALL blasts compared to brighter (nMFI, $p<0.0041$) and homogenous expression (CVIF, $p<0.0033$) in hematogones using Mann Whitney-U test. **Conclusion:**CD179α is strongly expressed in hematogones and widely expressed in B-ALL leukemic blasts. It can be a useful additional marker in lineage assignment, in challenging B-ALL cases with weak-CD19 expression or availability of only two B-cell markers. Due to its downregulated-expression, CD179a can also be a valuable marker for B-MRD assessment. However, more studies with large cohorts are needed to confirm our findings.

HEM-11

DISTRIBUTION OF TRBC1 IN T-CELL SUBSETS WITH SMALL FREQUENCIES AND ITS APPLICATION IN T-CELL NON-HODGKIN LYMPHOMA DIAGNOSIS AND STAGING

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Introduction: Establishing the clonality of T-cells is challenging in real-life practice, especially in samples with a small population of tumor cells. Recently, an antibody against the constant-1 region of β-chain of T-cell receptor (TRBC1) has been suggested to be a useful marker for clonality assessment due to its bi-modal distribution in major T-cell subsets. However, data on its distribution in subsets of T-cells with small frequencies in peripheral blood (PB) and bone marrow (BM) is not adequately available to use in staging samples with minimal involvement. We studied the distribution of TRBC1 expression in small subsets of T-cells in PB/BM from T-NHL submitted for staging and control samples. The results were validated using TCR-Vβ repertoire distribution levels.
Methods: We studied TRBC1-antibody (Clone-Jovi-1, fluorochrome-PE) along with other T-cell markers using 16-color assay (CD2/CD3/CD4/CD5/CD7/CD8/CD16/CD26/CD45/CD45RA/CD45RO/CD56/CD57/TCRγδ/HLADR/TRBC1) on FACS-LSR Fortessa. TRBC1 distribution was studied in T-cell subsets from 10-PB and 5-BM control samples and in 36 T-NHL patients. Results of TRBC1 were compared with TCR-Vβ repertoire distribution.
Results: 28 abT-cell subsets were evaluated for TRBC1+ distribution and their median/ranges are as follow: CD3T (31.53%; 21.33-49.36%), CD4T (37.67%; 29.78-52.60%), CD7-CD5+CD4T (35.26%; 20.65-55.98%), CD7+CD5+CD4T (36.23%; 29.06-47.55%), CD7-CD5-CD4T (25.82%; 16.66-41.69%), CD56+CD57-CD4T (38.25%; 29.33-54.05%), CD56+CD57+CD4T (40.09%; 28.57-62.79%), CD56-CD57+CD4T (45.50%; 16.55-88.22%), CD25+CD26-CD4T(37.57%; 28.57-61.00%), CD25-CD26-CD4T (34.40%; 21.59-46.10%), CD25-CD26+CD4T (36.18%; 24.43-56.94%), CD8T (24.60%; 14.13-38.74%), CD7+CD5+CD8T (25.40%; 15.83-34.08%), CD7+CD5-CD8T (27.75%; 13.04-56.60%), CD7-CD5-CD8T (29.42%; 18.18-63.51%), CD7-CD5+CD8T (24.67%; 14.93-44.77%), CD56+CD57-CD8T (24.15%; 14.24-48.70%), CD56+CD57+CD8T (29.24%; 13.84-75.54%), CD56-CD57+CD8T (24.80%; 14.12-41.96%), CD3+CD8+CD57+LGL (26.53%; 18.36-43.84%), CD4+CD8+T (40.21%; 23.90-58.03%), CD4-CD8-T (24.26%; 16.10-50.19%), CD45RA+CD3T (29.41%; 19.88-39.06%), CD45RA-CD3T (35.70%; 21.01-

49.90%), CD45RA+HLADR+CD3T (27.16%; 15.18-52.70%), CD45RA+HLADR-CD3T (28.19%; 21.80-33.71%), CD45RA-HLADR-CD3T (33.87%; 23.49-48.60%), CD45RA-HLADR+CD3T (35.93%; 23.99-56.76%). We also studied TRBC1 distribution in staging samples from 36 T-NHL cases, of which 35 patients (26.10% & 0.02-93.70%) showed immunophenotypic aberrancies and TRBC1 restriction and hence confirmed T-NHL involvement and reactive clonal expansion of cytotoxic T cells in 1 sample. In 26/36 samples, clonality of T cells was further confirmed by TCR-Vβ repertoire and in the remaining 10 patients, TCRVβ repertoire was not available.
Conclusions: We report the distribution of TRBC1-positive T-cells in 28 immunophenotypic subsets including those with small frequencies. We further confirmed its utility in the assessment of PB/BM involvement submitted for T-NHL staging and first-time reported its comparison with TCR-Vβ repertoire for clonality assessment.

HEM-12

UTILITY OF A MULTICOLOUR T-ALL MRD TUBE USING AN EXPANDED SURFACE ANTIGEN TUBE

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Introduction: Measurable residual disease (MRD) in T-cell acute lymphoblastic leukaemia (T-ALL) has been proposed as a powerful prognostic indicator for clinical outcome, however outside of clinical trials multicolour flow cytometry based T-ALL MRD has been somewhat limited with utilisation of immaturity markers such as CD99 and TdT. Recent consensus approaches to T-ALL MRD by immunophenotyping principally involves exclusion of normal T/NK cells to leave behind the immature T-ALL, if any. The MRD analysis approach is by using abnormal expression of myeloid antigens and T cell antigens such as CD2 and CD5; and the abnormal expression of antigens such as HlaDr, CD10 and CD1a. Here we look at combining a multicolour tube for surface markers with a second tube involving both cytoplasmic and surface markers.
Method: FCM analysis was performed on BM/PB samples using the following panels. Data were acquired on 12C BDFACSLyric and analysed on BDFACSSuite and Infinicyt software. **Tube1**: **Tdt**FITC, **cytCD3**PeCy7, **CD7**PE, **smCD3**PcPCy5.5, CD10APC, **CD8**APCcy7, **CD4**v450, **CD45**V500c, **CD56/CD16**BV605. **Tube2: CD99**FITC **CD7**PE **CD3**PcP5.5 **CD1a**APC **CD5**PECy7 **CD8**APCcy **CD4**v450 **CD45**V500c **CD56/**CD16BV605. **Tube3: CD5**FITC, **cytCD3**Pecy7, **CD7**PE, **CD34**PcPCy5.5, **CD13/CD33**APC, **CD117**APCCy7, **HLADr**v450, **CD45**V500c, **CD56/CD16**BV605. **New proposed Tube4: CD16/CD56/CD57/TCRgd**FITC, **CD7**PE, **CD1a**PECy5, **smCD3**APCH7, **CD8**APC, **CD99**BV605, **CD4**V450, **CD45**V500c, **CD34**BV786, **CD2**BV711, **CD5**PEcy7.
Results: With the initial 3 tube approach 8 T-ALL patients were studied for MRD. 21 x 3 tubes were processed at various time points. The results from these tubes showed the loss of applicability of tube2 in some patients. The exclusion gate was not sufficient and it has only one immaturity marker, CD99. Therefore we propose a 14 antigen 11 colour panel with the addition of CD2 and CD34. We also suggest a more comprehensive 'dump gate'. Further work with the additional tube was performed in 8 patients at 13 time points post Day0. The data showed the 6 out of 13 time points lost applicability with the Tube2, however the new proposed tube4 maintained its applicability (results were comparable to molecular testing).
Conclusions: The correlation between our proposed 14 antigen tube and the corresponding cytoplasmic tube is superior to the previous surface targeted approach. The combination of the 14 antibody surface tube with the appropriate cytoplasmic tube improves applicability of T-ALL MRD testing at treatment time points. It has (1) Bigger exclusion gate, (2) CD2 and CD5 together, (3) two immaturity markers CD99 and CD34

HEM-13

ROLE OF LAIR1 (CD305) IN FLOW CYTOMETRIC DETECTION OF OCCULT BONE MARROW INVOLVEMENT IN NON-CLL B-CELL NON-HODGKIN LYMPHOMA

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Introduction: Staging of B NHLs is crucial as it provides definitive evidence of the advanced stage of disease and important information for making a decision regarding treatment strategy. Bone marrow involvement is a vital part of staging B-NHL. We hypothesized that CD305, also known as LAIR1 (leukocyte immunoglobulin-like receptor-1), is a robust marker for detecting occult bone marrow involvement. LAIR1 is a transmembrane glycoprotein that acts as an inhibitor receptor and is expressed by most immune cells. LAIR expression varies during various stages of B cell differentiation.
Methods: CD305 (BV78, clone DX26) was incorporated in the lymphoma screening tube used for assessment of bone marrow (BM) involvement as a part of B-NHL staging between January 2020 and December 2021. Occult bone marrow involvement was defined as B-NHL involvement with <10% lymphoma cells. Multicolor Flow cytometric immunophenotyping (MFC) was performed using a 13-color panel on DxFlex flow-cytometer. Data were analyzed using Kaluza-V2.1-software.
Results: We studied BM samples from 1084 patients. Of them, 148 cases were clearly involved by morphological/immunophenotypic evaluation. Of the remaining, 172 cases did not reveal morphological involvement and were detected on MFC independently with a median tumor burden of 1.05(0.005-89). The median age was 57 years and the range was 7-80 years. These included CD5 positive lymphomas 35/172(20.3%), CD10 positive lymphomas 77/172(44.7%) and CD5, CD10 double-negative lymphomas 60/172, (34.8%). 149/172 (86.6%) cases revealed occult BM involvement. In 93/149 (62.4%) cases, B-NHL cells were identified using the expression pattern of CD5, CD10, CD11c, CD20, CD38, CD45, and Forward scatter in the background of normal B-cells. In the remaining 48/149(32.2%) cases, the tumor population was identified using only CD305 negative expression. Median(range) of tumor burden was 0.59% (0.016-9.7 %). Overall, CD305 was downregulated in 155/172 (90.1%) cases. CD305 was indispensable in the detection of occult BM involvement in 56(37.6%) cases which, lacked other immunophenotypic abnormalities, and was helpful in 99/172 (57.6%) samples.
Conclusion: CD305 is a crucial marker in detecting bone marrow involvement in cases of B-cell NHL by MFC, particularly CD5 and CD10 negative lymphomas where detection of minimal BM involvement would be challenging without the incorporation of CD305 in the lymphoma screening panel. It also provides an aberrant expression as an additional abnormality in a significant proportion of samples and provides supporting evidence in the detection of minute tumor populations

HEM-14

Utility of combination of anti-CD10, anti-PD1, anti-CXCR5, and anti-ICOS antibodies in the diagnosis/staging of Follicular-Helper T-Cell Lymphoma/Angioimmunoblastic T cell lymphoma

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Introduction: Follicular-helper T-NHL (FHTCL) or angioimmunoblastic T-cell Lymphoma (AITL) is a common subtype of T-NHLs. However, it is challenging to diagnose due to the lack of a unique histopathological pattern and low tumor burden with abundant inflammatory cells in the background. It is frequently misclassified and misdiagnosed. Flow-cytometric immunophenotyping(FCI) provides a unique ability to identify rare tumor cells. We studied utility of a 13-color FCI in diagnosis and staging of FHTCL/AITL.
Methods: A 13-colour FCI including antiCD10, anti-PD1 (CD279), anti-CXCR5 (CD185), and anti-ICOS (CD278) antibodies was performed in staging bone marrows (BM), peripheral blood (PB), FNA or body fluids (BF) samples. Tissue examination was performed by H&E and immunohistochemistry. The final diagnosis was made in accordance with WHO 2016 classification of hematolymphoid neoplasms.
Results: We diagnosed 54 cases (PB-3, BM-39, FNA-11, BF-2) of FHTCL/AITL with a median age of 58-years (range, 34-84 years) and M:F ratio-2.8. We observed a characteristic immunophenotypic signature of FHTCL/AITL cells with moderate-CD2 (87.0%), moderate-CD5 (92.5%), moderate-CD4 (85.1%), negative/dim-CD3 (85.1%), negative/dim-CD25 (62.9%), negative/dim-CD26 (85.1%), variable-CD38 (25.9%) and partial loss of CD7 (62%). Among four FHTCL/AITL associated markers studied, CD10 was heterogeneously positive in 38/51 (74.5%), PD1 was moderate in 40/45 (90%), CXCR5 weak positive in 20/33 (60.6%) and ICOS was moderate-weak positive in 10/11 (91%). On correlation with histopathological results, 11-cases were found to be misclassified as PTCL-NOS and 5-cases were misdiagnosed non-T-cell NHL (3-B-NHL, 1-classic Hodgkin lymphoma and 1-NLPHL) on histopathological evaluation. The diagnosis was corrected using FCI on lymph node-FNA and repeat tissue biopsy with additional IHC markers.
Conclusion: We report the utility of antiCD10, anti-PD1 (CD279), anti-CXCR5 (CD185), and anti-ICOS (CD278) in FCI diagnosis and staging of FHTCL/AITL. Our data confirmed the unique immunophenotypic signature of FHTCL/AITL. We report a critical role of FCI for correct diagnosis and staging in FHTCL/AITL, especially in samples with a low disease burden.

HEM-15

HYPOXIA SIGNALLING PATHWAY AND THE ENZYMES INVOLVED IN DNA DEMETHYLATION IN B-CELL ACUTE LYMPHOBLASTIC LEUKEMIA AND CHRONIC LYMPHOCYTIC LEUKEMIA PATIENTS

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Introduction: Literature data indicate a close relationship between low oxygen levels in cancer and epigenetic modifications. There are plethora of studies which support the link between DNA methylation/demethylation and carcinogenesis. Enzymes involved in this process are: TET (Ten-Eleven Translocation) and TDG (Thymine-DNA glycosylase). Loss-of-function mutations in TET proteins family have been described in various hematological malignancies. It is suspected that hypoxia has a significant influence on the regulation of activity/amount of TET enzymes. Thus, the aim of the study was to determine the expression of TET, TDG as well as HIF-1AN and HIF-1alpha in acute lymphoblastic leukemia (B-ALL) and in B-cell chronic lymphocytic leukemia (B-CLL) patients.
Method: The study included 9 children with B-ALL and 12 adults with B-CLL. Peripheral blood samples were collected into tubes containing EDTA and the cellular surface antigen-stabilizing agent. The blood samples were stained with direct conjugated anti-human antibodies (CD56/CD16-PE, CD19- APC-H7, CD45-BV421, CD3-V500, CD34-PE-Cy7) for identification of blastic cells (B-ALL), leukemic B-cells (B-CLL) and peripheral residual lymphocytes (B-, T-cells). Next, the cells were fixed, permeabilized and co-stained by indirect method with anti-human primary antibodies (HIF-1AN, HIF-1alpha, TET1, TET2, TET3, TDG) followed by compatible secondary antibodies conjugated with fluorescent dyes (donkey anti-rabbit Alexa Fluor-488 or rabbit anti-goat Alexa Fluor-647). The expression of analyzed proteins calculated as fluorescence intensity fold change over negative control.
Results: A significantly higher expression of TET1 and TDG was observed in leukemic B-cells in B-CLL comparison to blastic cells and residual B-and T-cells in B-ALL. There were no differences in the expression of TET-2 and TET-3 between the studied groups and populations of cells. HIF family enzymes expression in a population of blasts and leukemic B-cells was further analyzed. A significantly lower HIF-1AN expression was demonstrated in the group of patients with B-CLL vs. B-ALL with no differences in HIF-1alpha expression.
Conclusions: Literature data indicate a significantly reduced activity of TET family enzymes in B-CLL patients. On the other hand, the above studies showed a higher expression of TET1 in all studied cell populations of B-CLL vs. B-ALL patients. This may indicate an attempt to increase the enzyme activity by increasing its amount in the cells (compensatory mechanism). In addition, decreased HIF-1AN expression may indicate an increased hypoxia sensitivity of the tested cells, which in turn may regulate TET1 expression. This work was supported by the Excellence Initiative¬Research University, “Debut 2” and the University Center of Excellence Towards Personalized Medicine.

HEM-16

REDESIGNED LEUKAEMIA IMMUNOPHENOTYPING EXTERNAL QUALITY ASSESSMENT PROGRAMME - AN OVERVIEW OF PARTICIPANT RETURNS

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Introduction: The United Kingdom National External Quality Assessment Scheme for Leucocyte Immunophenotyping (UK NEQAS LI) has operated a leukaemia immunophenotyping external quality assessment (EQA)/proficiency testing (PT) scheme for over 30 years. The programme has undergone various changes culminating in a major redesign in 2021. Historically, participants were required to test for specific antigens (maximum 20) and report the percentage positive cells as well as classify antigens as positive/negative. The redesigned system requires participants to use the case history and digital image provided to determine which antigens to test for (no maximum), this system better reflects laboratory practice. Results are reported in terms of positivity/negativity and staining intensity for each antigen, together with details of the antibody combinations used in the analysis. This therefore provides an insight into how flow immunophenotyping is currently used to diagnose leukaemia.

Methods: We report findings from the first 12 months of the redesigned Leukaemia Immunophenotyping (LI) Part 1 programme. Data from all 6 cases were analysed to identify the top 10 commonly tested antigens for the disease types issued and identify the number of participants testing for these. The disease types issued were B-acute lymphoblastic leukaemia, B-chronic lymphocytic leukaemia (x2), mantle cell lymphoma, acute myeloid leukaemia with mutated *NPM1*, and acute monoblastic and monocytic leukaemia. Analysis was also undertaken to identify any participants using identical panels for specific disease types and finally, the level of consensus for individual antigens tested for within each case.
Results: An average of 49% of participants were found to test for the top 10 antigens in each of the 6 cases issued (41-62%). Very few participants (less than 1% for 5 of the 6 cases and 6% for the remaining case) were found to be using identical panels. For individual antigens tested for within each case, testing rates varied from 76.4% (CD79b) to 100% (CD19), with an average of 91.7% for the top 10 antigens analysed in each case. 100% agreement was only seen twice over the course of the exercises examined.
Conclusions: Whilst there was broad consensus in core aspects of panel design, most participants were found to be using bespoke panels when compared to other laboratories. Consensus for individual antigens tested for within the specific cases was rare, which raises concerns over inclusion/omission of key antigen testing in leukaemia immunophenotyping. Overall, the findings highlight a lack of standardisation in flow cytometry for leukaemia testing.

HEM-17

PD-L1 EXPRESSION IN MYELOID-DERIVED CIRCULATING CELLS AS A POTENTIAL BIOMARKER TO DETERMINE TREATMENT RESPONSE TO CANCER IMMUNOTHERAPY

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Introduction: Programmed Cell Death Protein 1/Programmed Cell Death Protein Ligand 1 (PD-1/PD-L1) is an immune axis widely targeted in immunotherapy. Non-Small Cell Lung Cancer (NSCLC) treatment is improved by five anti-PD-1/PD-L1 approved immunotherapies. Treatment decision-making relies on quantification of PD-L1 levels in tumor tissue which are used to calculate the Tumor Proportion Score. Thus, an accurate identification of PD-L1 is crucial to optimize treatment administration. In our study, we have prospectively studied PD-L1 expression in Myeloid-Derived Suppressor Cells (MDSCs) from peripheral blood of NSCLC patients undergoing anti-PD-1/PD-L1 immunotherapy.
Methods: NSCLC patients (n=40) were classified based on clinical outcome: tumor reduction (responders, n=15), tumor stabilization (partial responders, n=8), tumor progression (non-responders, n=10), and non-assessable (*exitus*, n=7). Peripheral blood samples were collected in EDTA-anticoagulated tubes, prior to and during anti-PD-1/PD-L1 immunotherapy. Then, whole blood was immediately processed using a minimal sample perturbation protocol developed by our group (Rico et al., 2021). Briefly, blood was stimulated with PMA, which results in a conformational change of PD-L1 and epitopic target accessibility. MDSCs were identified according to HLA-DR$^{lo/-}$CD33^{+}CD11b^{+} immunophenotyping and analyzed on the Attune™ NxT flow cytometer (Thermo Fisher). FCS files were analyzed with OMIQ and FlowJo™ (v.10) and data displays were generated with GraphPad Prism (v.9).

Results: MDSCs populations were computationally analyzed using FlowSOM clustering and used to generate self-organized maps characteristic of each clinical outcome group (non-responders, partial responders, responders, and *exitus*). Individual maps revealed specific profiles and were assigned to each group, and a non-significant increase of PD-L1 stain index was observed with tumor progression. **Conclusion:** Unstimulated MDSCs showed no PD-L1 conformational change. After PMA stimulation, PD-L1 evidenced drastic conformational changes and epitopic targeting, allowing PD-L1 expression in peripheral blood MDSCs to be measured with this rapid and non-invasive assay, as a promising biomarker of the immune status of each single patient. Personalized immune monitoring using clustering analysis may provide a new experimental approach to identify disease-associated immune signatures and its association with risk-progression in NSCLC.

HEM-18

DIAGNOSTIC USEFULNESS OF CD180 EXPRESSION BY FLOW CYTOMETRY IN BLPD ANALYSIS

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Introduction: Flow cytometric assessment of BLPD examines a range of CD targets which combined with other test modalities usually ensures confidant diagnosis and assignment to WHO entity and subsequently appropriate treatment. Some diagnostic overlap between entities exists however, so development of the repertoire of markers used is desirable. CD180 expression was suggested to be conserved on MZL compared with other entities. We looked to confirm its utility in our local current and historic cases, confirm expression on stored samples and to elucidate its expression in subclinical cases; MBLs.
Methods: Excess material from routine investigations allowed paired fresh and DMSO stored blood, marrow and spleen samples containing a wide range of clonal B cell entities to be assessed using local standard Lyse wash protocols with data acquired using a FACSCantoII (BD). A panel (CD45, CD19, CD5, CD180, +/- CD103) was established for efficient identification of relevant populations with CD180 MFI as the primary measurand. T cell, polyclonal B cell and monocyte populations provided internal controls. The extended diagnostic phenotype and the assigned WHO entities were utilised to group the patients for comparison of CD180 MFI.
Results: Paired fresh EDTA and LiHep and paired fresh and DMSO stored samples showed comparative CD180 expression levels. MFI expression of CD180 on normal control populations showed no significantly different expression across diagnostic groups. No significant differences of CD180 expression were seen between CLL, SLL, LPL, MCL and CLL-like CD5+MBL ; or between SMZL, CBLMZ, and HCL. Significantly different CD180 expression levels were obtained for LPL vs SMZL ($p=0.0007$), and LPL vs CBLMZ ($p=<0.0001$) using unpaired T tests.
Conclusions: Demonstration of maintained CD180 expression on stored samples vs fresh samples allowed additional analysis of historic cases. Analysis of CD180 expression on B cell neoplasms showed differentiation between HCL/MZL cases vs other diagnoses confirming previous reports. Furthermore differential expression is also seen in the CD5-MBL group which largely correlates with CBLMZ vs other diagnoses when other test results were considered, e.g. MYD88, BRAFV600E, IGHV usage. Expression levels within control populations suggest refinement of the CD180 MFI measurand using a T cell and/or monocyte ratiometric method may better assess expression when compared to outright MFI. In conclusion CD180 should be considered for inclusion in B cell panels and may show particular use in instances of phenotypic overlap not otherwise clearly meeting diagnostic criteria.

HEM-19

CD72, CD44 AND MIB1 EXPRESSION AS INDICATORS OF RECURRENCE FREE SURVIVAL IN FOLLICULAR LYMPHOMA

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Introduction: Follicular lymphoma (FL) is the second most common type of B-cell non-Hodgkin Lymphoma in Western countries. It is characterized by a wide heterogeneity in onset, histopathology and disease course. According to the ESMO guidelines, treatment decisions range from watch-and-wait to immunochemotherapy (ICHT). The heterogeneity of FL is highlighted by the difficulty of identifying clinical and biological parameters that can predict clinical outcome and treatment efficacy. The diagnosis is based on cytomorphology, immunophenotype ($CD10^{+}Bcl2^{+}Bcl6^{+}$) and detection of t(14;18). Although flow cytometric immunophenotyping is rarely used for lymphoma characterization, in a previous study we showed that Artificial Intelligence models, applied to cytometric data, lead to a classification of different types of lymphoma. In this study, we evaluated whether immunophenotypic markers may correlate with clinic in a homogeneous group of FL patients.
Methods: We selected 53 FLs treated with ICHT (R-CHOP or R-Bendamustin) and for whom a follow-up ≥24 months was available. All cases were extensively characterized by multiparametric flow cytometry.
Results: The relapse-free survival analysis showed that, in addition to the Follicular Lymphoma International Prognostic Index (FLIPI), markers correlating with relapse were MIB1>16% ($p<0.03$), CD44>76% ($p<0.02$) and CD72>67% ($p<0.008$) (Wilcoxon test). The most powerful was CD72, which also positively correlates with FLIPI in multivariate analysis ($p<0.05$).
Discussion: This study suggests that increased expression of phenotypic markers correlates with FL clinical course, particularly with likelihood of post-therapy recurrence: (i) proliferative index (MIB1), known to associate with a higher histological grade; (ii) CD44, cell surface adhesion receptor expressed on normal and tumor cells, whose interaction with the extracellular matrix promotes metastatization; (iii) CD72, considered a positive regulator of B lymphocyte functions in patients with autoimmune diseases. Of these, CD72 is the more intriguing; considering its role in B lymphocyte, indeed, its overexpression in FL likely sustains increased proliferative activity and/or confers resistance to ICHT agents.

HEM-20

EXPRESSION PATTERN OF GL7 IN DIFFERENT STAGES OF B-CELL MATURATION AND ITS UTILITY IN B-LYMPHOBLASTIC LEUKEMIA/LYMPHOMA MEASURABLE RESIDUAL DISEASE ASSESSMENT

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Introduction: With the increasing use of targeted therapies such as anti-CD19 Blinatumomab/CAR-T cells and anti-CD20 Rituximab, additional B-cell maturation-associated markers can be helpful in evaluating deviation-from-normal in bone marrow (BM). Herein, we studied role of GL7 in assessing BM B-cell maturation and evaluated its utility in B-ALL patients including role in MRD assessment.
Methods: Expression of GL7 was studied on leukemic blasts in 97 B-ALL patients at baseline and on hematopoietic cells including B-cell precursors (BCP) from 10 uninvolved staging BM samples using 16-color MFC. Expression-level of GL7 was studied using mean fluorescent intensity (MFI) and expression-pattern (heterogenous/homogeneous) using CV of immunofluorescence (CVIF). Its expression was also studied in precursor B-cells and mature B-cells on Kaluza-v2.1 software. Further, GL7 expression was studied in 41 end-of-induction (EOI=D35) B-MRD samples to evaluate its utility in MRD assessment.
Results*:* Median (range) of GL7-positive percentage and MFI in CD34(+) early B-cell precursors (BCP-1) were respectively 0.75%(0-5.1%) and 0.88(0.23-1.81); in CD34(-) late B-cell precursors (BCP-2) were 69.39%(24.87%-99.14%) and 3.09(0.18-12.13); in mature B-cells were 91.5%(73%-98.7%) and 31.87(9.49-49.12). Thus, GL7 showed negative expression in BCP1, heterogenous/dim-negative in BCP2 and brightest in mature B-cells. Among non-B cells, a distinct proportion of T-cells (median-66.3%; range-39.3%-87.1%) and neutrophils (median-65.9%; range-64.4%-92.0%) expressed GL7. A cut-off level of greater than 5% was used to define positive GL7 expression in B-ALL. 77/97 (79.4%) of B-ALL demonstrated GL7-positive expression with median 65.6% and range 6.5%-99.93% in B-ALL blasts. However, its expression level was dim-to-heterogeneous (MFI- median-14.1 and range-1.3-35.6). EOI-MRD was available in 41/97 patients and 18/41(43.9%) patients had detectable MRD (median-0.024%; range-0.0002%-42.3%). 7/18(38.9%) samples showed increased percentage of blasts expressing GL7 and 11/18(61.1%) showed decreased percentage of blasts. We studied the effect of treatment induced changes in expression of GL7 and found overexpression as compared to the diagnostic sample in 14/18(77.7%) residual blasts in MRD+ patients with median over expression of 1.86 times.
Conclusions: GL7 appears in B-cell maturation in BM at late BCP2 stage and is highly expressed in majority of B-ALL samples at diagnosis. GL7 is overexpressed in post-treatment MRD blasts and provides a useful additional marker for the assessment of B-ALL MRD. It may be also useful as a potential therapeutic target for immunotherapy.

HEM-21

ASSESSMENT OF THE IMMUNE STATUS IN THE ASCITES FROM PATIENTS WITH HIGH-GRADE SEROUS OVARIAN CANCER

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Introduction: High-grade serous ovarian cancer (HGSOC) is the most lethal gynecological cancer, often characterized by presence of ascites fluid at the initial diagnosis. Ascites represents a reflection of the HGSOC tumor microenvironment and may serve as an additional source to assess the immune status. However, precise analysis of the immune cell populations present in the ascites and their expression of immune checkpoints remains under-researched. Our objective was to evaluate the exact proportion of the immune cell populations and determine the expression status of the programmed cell death protein 1 (PD-1) and its ligand (PD-L1) in the ascites from patients with primary HGSOC.

Methods: Ascites was collected from 31 patients diagnosed with primary HGSOC. The proportion of T-cells ($CD3^+$) and their subsets: epithelial-like T-cells ($CD3^+CD103^+$), cytotoxic T-cells ($CD3^+CD8^+$), helper T-cells ($CD3^+CD4^+$), regulatory T-cells ($CD3^+CD4^+CD25^+CD127^{\pm}$), NK-cells ($CD3^-CD16^{++}CD56^+$), B-cells ($CD19^+$), dendritic cells ($lineage^-HLADR^+CD123^+CD11c^+$), monocytes ($CD11b^+CD14^+CD68^-$) and macrophages ($CD11b^+CD68^+$), as well as PD-1 and PD-L1 expression was analyzed using flow cytometry. The study was approved by the Institutional Ethics Committee of Institute of Oncology Ljubljana and the National Ethics Committee Ljubljana, Slovenia (ERID-EK/170 and 0120-33/303/2018/3). All samples were obtained after appropriate informed written consent.
Results: The analysis of the immune status from the ascites showed predominance of T-cells (49±21), medium presence of NK-cells (25±17%) and monocyte-macrophages (24±20), and low presence of dendritic cells (1±2%) and B-cells (6±9%). Among the T-cell subsets, we observed predominance of the helper T-cells over the cytotoxic T-cells (56±16% and 53 ±16%, respectively) with similar expression rate of PD-1 (25±11 and 23±12%, respectively), and low presence of regulatory T-cells (11±7%) and epithelial-like T-cells (7±6%), with highest expression of PD-1 (45±25% and 51±15% respectively). Regarding NK-cells, three main subsets were detected: $CD16^+$ (18±7%), $CD16^+CD56^+$ (4±4%) and $CD56^+$ (4±2%), with PD-1 expression changing from highest to lowest, respectively (35±27%, 19±20%, 10±17%). Moreover, we observed a monocyte-macrophage transition, where $CD14^+$ monocytes constituted 13±13%, and $CD14^+CD68^+$ macrophages 10±9%, expressing similar level of PD-1 (19±18% and 21±18%, respectively). Dendritic cells and B-cells showed lowest PD-1 expression among the other immune cell populations (10±8% and 3±6%, respectively). Expression of PD-L1 was not detected at any cell population/subset.
Conclusion: We identified the presence of different immune cell populations and PD-1 and PD-L1 expression in primary HGSOC ascites. Data obtained highlights the impact of the flow cytometry as a promising diagnostic tool for HGSOC, with the opportunity to identify novel prognostic markers.

HEM-22

A NEW METHOD FOR THE AUTOMATED ENUMERATION AND ANALYSIS OF CD34+ HAEMATOPOIETIC STEM AND PROGENITOR CELLS BY FLOW CYTOMETRY

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Introduction: Clinical laboratories rely on commercially available IVD solutions for CD34+ cell enumeration to avoid time- and resource-consuming validation of user-defined tests. Most reagent kits and software packages used in flow cytometry were developed as a response to the 1996 and 1998 ISHAGE Guidelines but have not been updated since then to meet the growing demands of diagnostic laboratories, in terms of automation, data traceability, and the ability to adapt acquisition and analysis panels to evolving regulations and guidelines.
Methods: We have developed* an automated solution for CD34+ cell enumeration by flow cytometry, consisting of hardware, software, and reagents, that streamlines operations by incorporating automated sample loading, sample preparation, reagent management, and barcode scanning, as well as data analysis and bidirectional LIS connectivity in one compact device platform.
Results: Samples are loaded on the system by the operator, and sample preparation and data analysis are performed automatically by the analyser. The acquisition and analysis software follows the sequential gating strategy of the ISHAGE Guidelines and provides panel options that allow the running of tests in duplicate plus negative control, without the use of a negative control, or as a single test.

Antibody and reagent vials include a unique barcode identity for tracking the expiration date, onboard expiration, manufacturing lot and container numbers, supporting Quality Management system data traceability requirements.
Conclusions: Our newly developed system for CD34+ cell enumeration by flow cytometry provides a comprehensive solution for automated CD34+ cell enumeration that minimizes the need for human intervention, and potentially enables laboratories to offer CD34+ enumeration outside regulatory lab office hours. Both innovative aspects may increase the level of patient care and reduce time-to-result for this time-critical application.
* Pending submission and clearance by the United States Food and Drug Administration; not yet available for in vitro diagnostic use in the US. For Investigational Use Only. The performance characteristics of this product have not been established. Patent pending.

HEM-23

EVALUATION OF CD81 EXPRESSION IN HEMATOGONES AND IN LEUKEMIC B LYMPHOBLASTS

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Introduction: The distinction of leukemic B Lymphoblastic Leukemia (B-LBL) blasts from normal bone marrow B cell precursors, i.e hematogones (HG), by multiparameter flow cytometry (MFC) can be challenging due to the presence of common immunophenotypic markers shared by both cell types. CD81 has more recently emerged as one marker to be used in the assessment of Minimal/Measurable disease (MRD). Only a couple of publications have addressed this subject. The purpose of this study is to evaluate the usefulness of CD81 in the context of B-LBL versus Hematogones.
Methods: A total of 105 bone marrow samples were analyzed with a multiparameter flow cytometry 10 color panel using the following 10 markers: CD10, CD19, CD20, CD34, CD38, CD45, CD66c, CD73, CD81, and CD123. These 105 samples consisted of 60 B-LBL cases and 45 samples negative for B-LBL but with hematogones. The mean fluorescence intensity (MFI) of CD81 was recorded in B-LBL blasts and in Hematogones. Statistical comparison between lymphoblasts and the different stages of hematogone maturation was carried out using t-test.
Results: 88.5% of B-LBL cases displayed an abnormal expression of CD81 respectively when compared to hematogones. A statistically significant distinction between hematogones (stages 1,2 and 3) from lymphoblasts was established by comparing MFI of CD81 ($P<0.0001$). Nevertheless, the overlapped expression between the two cell populations was high at 38%. However, a specific reproducible maturational pathway was observed for the different stages of hematogones when CD81 was plotted against CD45. Both CD81 and CD45 displayed different levels of expression in the various stages of HG maturation. This combination of both markers in the assessment reduced the overlap between B-LBL and HG, leading to a better distinction when using a combined Leukemia associated phenotype and different from the normal approach.
Conclusion: CD81 marker appears to be very efficient in clearly discriminating hematogones from neoplastic B lymphoblasts. Hematogones display a specific reproducible maturational pathway when CD81 is plotted against CD45. This combination results in excellent separation of neoplastic blasts from normal B cell precursors.

HEM-24

ABCB1 AS A TARGET OF MIDOSTAURIN IN ACUTE MYELOID LEUKEMIA

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Introduction: Patients diagnosed with acute myeloid leukemia (AML), a severe hematologic malignancy with poor survival rate, often don't meet satisfying therapy outcomes resulting in relapse or disease progression. The anthracycline-based induction therapy can be affected by a resistant phenotype associated with overexpression of ABCB1 transporters. Midostaurin, a multikinase inhibitor approved for the treatment of FLT3-mutated AML, has been identified as ABCB1 inhibitor.
Methods: In 28 primary AML samples isolated prior to any treatment, *ABCB1* gene expression was established by absolute quantification and ABCB1 efflux activity using ddPCR and flow cytometry, respectively. Results were compared to multiple clinical parameters such as mutations, ELN risk classification, complete remission or CD34 expression. ABCB1 regulation by microRNAs was evaluated as well as proapoptotic effect mediated by midostaurin which was tested in ABCB1-overexpressing leukemic cells.
Results: High *ABCB1* transcripts were found to be associated with unachieved complete remission, adverse ELN risk group and CD34 positivity. In functional study employing mitoxantrone and midostaurin, an increased mitoxantrone accumulation was observed in AML patients positive for CD34 marker and those not achieving complete remission while the presence of FLT3 and/or NPM1 mutations did not seem to play a role. Furthermore, induction of apoptosis was observed when leukemic cells were treated with daunorubicin and midostaurin. Moreover, ABCB1 efflux activity and gene expression were found to be directly linked to downregulation of miR-9 while no relation to miR-27a and miR-331 was observed.
Conclusions: We highlight therapeutic value of midostaurin in the treatment of drug-resistant AML patients and propose its beneficial use even in FLT3-negative patients. We also propose miR-9 as a predictive ABCB1-associated biomarker that might be employed in identifying ABCB1-resistant phenotype in AML and eventually help optimize therapy regimen.
Study was supported by SVV 260 549, PRIMUS 20/MED/010 and GAUK 205019.

HEM-25

CD19 NEGATIVE PERITONEAL PROGRESSION IN PATIENT WITH DLBCL AFTER CAR-T THERAPY

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Introduction: A 55-year-old male patient with DLBCL received CAR-T therapy (tisagenlecleucel) as third line therapy. At the time of the infusion, the patient presented with an abdominal bulky enveloping the superior mesenteric vessels, bone marrow biopsy was negative for disease. He began to progress 30 days after receiving CAR-T therapy (tisagenlecleucel), with a mesenteric, retroperitoneal, hepatic perihepatic disease progression which caused an ascitic effusion.

Methods: We analyzed by flow cytometry the ascitic effusion. The immunophenotype evaluation was performed on fresh sample with a panel of directly conjugated antibodies (kappaFITC-lambdaPE-CD19ECD-CD5PC5.5-CD20PC7-CD14APC-CD23A700-CD10A750-CD2PB-CD45KO, Agilent and Beckman-Coulter). The analysis were performed using a Navios cytometer (Beckman Coulter) and Navios software. The analysis of lymphocyte subpopulations was performed on lymphocyte population gate, using quadrant dot plot statistics. Immunomonitoring after infusion was performed on day +3, +7, +10, +15, +30, +45 using a quantitative count with single platform analysis with a ten color panel (CD16FITC-CD127PE-CD14ECD-CD25PC5.5-CD4PC7-CD56APC-CD19A700-CD3A750-CD8/CD21PB-CD45KO)The kinetics of CAR-T expansion in peripheral blood was evaluated at the same time points and CAR-T evaluation on ascitic effusion was made (CD19 CAR Detection Reagent, Miltenyi).
Results: Before CAR-T infusion, the histological examination of the lymph node tissue showed large immunoreactive lymphoid elements for CD20, bcl-6, bcl-2 and CD10. After lymphodepleting therapy with fludarabine and cyclophosmide, he received CAR-T therapy. The course was complicated by CRS G1, for which 2 doses of tocilizumab were administered and then, the appearance of CRS G2. Symptoms resolved 6 days after infusion. The kinetics of CAR-T expansion showed lower concentration: 3 CAR-T/microl at day +3 and 4/microl at day +7 and values close to zero in subsequent time points. At progression the ascitic effusion contained B cells with high FSC, and positive expression of CD20, CD10, surface kappa light chain and negative for CD19. CAR-T in ascitic effusion were undetectable.
Conclusions: Despite frequent durable remissions induced by CART in hematologic malignancies, about 60% of patients show a lack of response or eventually relapse. The mechanisms of this resistance have been mainly investigated in ALL but remain scarcely understood in DLBCL. The impact of CD19 loss in DLBCL remains unclear. From a practical standpoint, it must be stressed out that CD19 immunostainings would be relevant in histological evaluation before CAR-T therapy. The possibility of performing the immunophenotypic profile with flow cytometry, would make even easier to evaluate the expression of CD19, in the study of CAR-T resistance.

IMM-01

CHANGES IN B-CELL SUBTYPES IN COVID-19 AND THEIR RELATIONSHIP TO DISEASE SEVERITY

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Introduction: COVID-19 disease is a respiratory tract infection caused by the new coronavirus, called SARS-CoV-2. It is believed that the evolution in the severity of the disease, as well as the resistance to its development, is related to the individual immune response of the infected, since SARS-CoV-2 infection activates the innate and adaptive immune responses. Thus, the aim of this study was to evaluate the frequencies and absolute numbers of B-lymphocytes and their subtypes in patients with COVID-19, in order to clarify their relationship with peripheral immune system changes and disease severity.
Methods: This study included 133 COVID-19 patients and 30 healthy controls, who attended at the University Hospital of the Federal University of Santa Catarina and Nereu Ramos Hospital, Brazil. Patients were classified according to disease severity as mild (n=36), moderate (n=30), severe (n=32) and critical (n=35). PB mononuclear cells (PBMCs) were isolated using Ficoll-Hypaque and subdivided by flow cytometry into naïve, transitional, non-switched memory B-cells (NS-MBCs), class-switched memory B-cells (CS-MBCs) and double negative (DN) B-cells.
Results: When investigating the B-cell compartment of COVID-19 patients, a reduction in the relative value of B cells was observed when compared to the control ($P < 0.001$). In addition, it was observed a reduction in the absolute count of transitional cells ($P = 0.024$) and in both relative and absolute count of NS-MBCs ($P < 0.001$), CS-MBCs ($P < 0.001$) and plasmablasts ($P < 0.001$). All of these changes were seen in patients with moderate to critical COVID-19, when compared to the control group. When evaluating the frequencies of DN B-cells, an increase in the absolute ($P < 0.001$) and relative ($P = 0.001$) value of these cells was observed in patients with mild and critical COVID-19.
Conclusions: The results suggest that the B-cell compartment abnormalities are associated with clinical worsening in COVID-19 patients, which could serve as a possible prognostic marker in patients with COVID-19.

IMM-02

DIFFERENTIATED EXPRESSION OF PD-1 AND CTLA-4 IN PERIPHERAL BLOOD T LYMPHOCYTES OF PATIENTS WITH BREAST CANCER

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Introduction: The T-lymphocyte is the most important cell in antitumor defense and the expressions of PD-1 (programmed cell death protein 1) and negative co-stimulatory molecule CTLA-4 (cytotoxic T-lymphocyte-associated protein 4), present on the surface of T cells, are critical regulators of its function in diverse processes ranging from autoimmunity, transplant tolerance, chronic viral infections, and tumor immunity. CTLA-4 limits T cell activity during immune responses, and PD-1 reduces T cell activity in peripheral tissues. The expression of these proteins has been important in the evaluation of the immune response in breast cancer (BC), but associations between these biomarkers and tumor subtypes have not yet been investigated. Thus, the objective of this study was to evaluate the prognostic value of PD-1 and CTLA-4 biomarkers in different subtypes of BC.
Methods: Peripherical blood samples from 62 patients previously diagnosed with BC were labeled with antibodies against CD279 (PD-1, PE), CD152 (CTLA-4, PE-Cy7), CD4 (PB) e CD8 (FITC) and CD45 (PO) and analyzed by flow cytometry (FC). The expression of RE, RP, HER2 markers in tumor samples from surgical specimens was analyzed by Immunohistochemistry (IHC) to determinate BC subtypes, based on 13th Gallen Conference.
Results: When compared according to the malignancy of the tumor (malignant vs benign), it was possible to observe that TCD4 lymphocytes showed a reduction in the expression of CTLA-4 (P = 0.045) and PD-1 (P = 0.001) in the malignant samples. The same result was observed for TCD8 lymphocytes, which showed a significant reduction in these markers (P = 0.024 and P = 0.016). This comparison was also made by subdividing BC samples into luminal A, luminal B, HER2 overexpressing, and TBNC (triple-negative breast cancer). Patients with luminal A BC showed reduced PD-1 expression in TCD4 (P = 0.032) and TCD8 (0.002) cells when compared with benign samples. As for CTLA-4, a reduction in the expression of this protein was observed in TCD8 cells of HER2+ tumors (P = 0.021) when compared with benign samples.
Conclusions: TCD4 and TCD8 lymphocytes in the peripheral blood of BC patients showed reduced expression of CTLA-4 and PD-1 biomarkers, especially, reduced PD-1 of TCD4 in luminal A BC patients and of CTLA-4 inTCD8 cells in HER2+ tumors.

IMM-03

T CELL PHENOTYPES AND THEIR RELATION TO VITAMIN D LEVEL IN ATOPIC DISEASES

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Introduction: Immune response in atopic diseases could be associated with different T cell phenotypes. Moreover, vitamin D may have a relation with T cells immune response. The relationship between T cell phenotypes and vitamin D in atopy has not well-defined. The aim of the study was to evaluate T cell phenotypes and their possible relation with vitamin D level in subjects with atopy.
Methods: In total, 30 subjects with atopy (15 with mild to moderate atopic dermatitis – AD, 15 with mild to moderate allergic asthma – AA) and 15 age-matched healthy subjects who do not take vitamin D supplements were involved in the study. Peripheral blood mononuclear cells were stained with monoclonal antibodies (anti-CD25–CD4–FoxP3 for Treg, anti-CD4–IL-17A–INF-GMA–IL-4 for Th1/Th2/Th17 cells) and evaluated using flow cytometry. Serum 25-hydroxy vitamin D level was evaluated by ELISA. According to the recomendations, subjects were divided into groups based on their vitamin D level: severe deficiency <20 nmol/l, deficiency 20-50 nmol/l, insufficiency 50-75 nmol/l, normal amount 75-200 nmol/l.
Results: Significantly lower percentages of Treg cells were detected in subject with AA compared to AD or control groups (1.56 ± 0.15 vs 2.34 ± 0.26 and 2.35 ± 0.16 %; respectively, $p<0.05$). In subject with AD, relative numbers of Th17 cells were higher than in control group (0.96 ± 0.15 vs 0.54 ± 0.06 %; $p<0.05$). AD and AA groups had significantly higher proportion of Treg and Th17 cells compared to control group (0.49 ± 0.09 and 0.47 ± 0.08 vs 0.25 ± 0.03; respectively, $p<0.05$). In subjects with atopy there was a positive correlation between Th2 and Th1 cells ($r=0.57$, $p=0.001$), Th2 and Th17 cells ($r=0.37$, $p<0.05$). Negative significant correlation was established between Th1/Th2 proportion and vitamin D level in subjects with atopy ($r=-0.38$, $p<0.05$). There was no significant difference of vitamin D levels between the groups. In subject with atopy and vitamin D severe deficiency were detected significant higher proportion of Th1/Th2 cells compared to the groups of atopic subject with vitamin D deficiency, insufficiency or normal amount (0.45 ± 0.25 vs. 0.19 ± 0.16; 0.14 ± 0.5; 0.21 ± 0.3; respectively, $p<0.05$).
Conclusions: Decrease of Treg and increase of Th17 cells as well as association between Th2/Th1 cells and vitamin D in subjects with allergic asthma and/or atopic dermatitis let us hypothesize about an important role of different T cell phenotypes and vitamin D in the pathogenesis of diseases related to atopy.

IMM-05

FLOW CYTOMETRIC ANALYSIS OF PD-1 EXPRESSION IN PERIPHERAL T CELLS OF PATIENTS WITH IDIC-15, A NEUROLOGICAL RARE DISEASE

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Introduction: Idic15 syndrome is a rare genetic pathology of neurodevelopment caused by variable duplications in the q11-q13 gene region of chromosome 15. Its symptoms are very heterogeneous but similar to that of other neurodevelopmental pathologies and include enhanced susceptibility to infections in many patients. In previous studies by flow cytometry (FCM) we have shown immunophenotypic alterations in several subpopulations of T, B and NK cells. Programmed Cell Death-1 (PD-1) is a co-inhibitory receptor involved in the regulation of T-cell activation, differentiation, effector function and memory. PD-1 signaling limits the activation of self-reactive T cells, reducing the risk for autoimmunity. However, in chronic infections, sustained PD-1 expression may impair protection against pathogens.
Methods: We have studied by FCM the expression of PD-1 protein in helper (CD4) and cytotoxic (CD8) peripheral T-cells in a cohort of Spanish patients with Idic-15 syndrome and an age- and sex-matched group of healthy individuals.
Results: Our data showed no significant difference between controls and patients in either the absolute and relative abundance of circulating CD4 or CD8 cells expressing PD-1 or the mean intensity of PD-1 expression per cell. Interestingly, PD-1 expressing CD4 (p=0.02) or CD8 (p=0.08) cells were more abundant in women than in men in both groups. Moreover, we found an age-dependent decrease of the intensity of expression in PD-1 positive CD4 cells in both controls and patients, independent of their gender. Regarding the role of impaired PD-1 expression in infections, we found lower numbers of PD-1 expressing CD4 (p=0.09) and CD8 (p= 0.01) cells in Idic-15 patients with repeated infections, together with a non-significant decrease of PD-1 expression per cell. No significant changes in PD-1 FCM parameters were observed in subgroups of patients stratified acording to their genetic lession or main neurological manifestations.
Conclusions: Our findings suggest that PD-1 expression may change according to age and sex while, independent of these variables, PD-1 signaling may be involved in the immune alterations of Idic-15 patients. Sponsored by donations to the “One House One Life” Initiative promoted by Great Chance SLU.

IMM-06

FLOW CYTOMETRIC OX40 ASSAY ANALYSIS TO DETERMINE SPECIFIC SARS-COV-2 T CELL RESPONSES

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Introduction: Immunocompromised individuals (transplantation, autoimmunity, oncology) and patients with inborn errors of immunity (IEI) are at increased risk of severe coronavirus disease-2019 (COVID-19). Effective vaccination against SARS-CoV-2 is therefore of great importance in this group, but the immunogenicity of SARS-CoV-2 vaccines can be uncertain. Plasma serology is often not sufficient to make an estimation of immunization in these patients. So measuring cellular immunity to SARS-CoV-2 vaccine is of importance. The so-called ‘OX40’ assay, a flow cytometry method based on detection of CD25 and OX40 (CD134) surface membrane expression of activated CD4+ T cells, is a rapid and robust whole blood technique that is concordant when compared with more traditional methods of antigen-specific T cell identification.
Methods: In the present study more than 50 immunocompromised or IEI patients were included that received at least 2 doses of the SARS-CoV-2 vaccine. The ‘OX40’ assay to detect antigen-specific T cell responses to the S-protein of SARS-CoV-2 was used. Whole blood of the study subjects was stimulated with S-protein spanning peptide pools and after 44-48 hr cells were harvested, stained and acquired by flow cytometry.

Results: More than 50 immunocompromised individuals were tested for antigen-specific T cell responses to SARS-CoV-2 after vaccination. About 90% of the tested individuals showed a normal response as compared with vaccinated healthy controls. In the remaining 10% we detected an absent or low response after vaccination.
Conclusions: The OX40 assay allows monitoring of the magnitude and duration of functional T cell immunity to SARS-CoV-2, that can help in prioritizing revaccination strategies in vulnerable populations.

IMM-07

NEUTROPHIL LYMPHOCYTE RATIO (NLR) AND PB MYELOID DERIVED SUPPRESSOR CELLS (MDSC) FREQUENCY IN PATIENTS WITH METASTATIC CASTRATION-RESISTANT PROSTATE CANCER (mCRPC)

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Introduction: The Neutrophil Lymphocyte Ratio (NLR) obtained from peripheral blood (PB) full blood count is a negative prognostic marker in prostate cancer (PCa) and other cancers. Myeloid derived suppressor cells (MDSC) are a pathologically differentiated myeloid cells of granulocytic and monocytic origin expanded in patients with cancer and other diseases. These cells are immunosuppressive and promitogenic. The aim of this study was to characterise PMN and M-MDSCS in the blood of men with mCRPC and to determine the relationship between neutrophil, lymphocyte and NLR from routine hematology and circulating MDSC frequency.
Methods: Peripheral blood was collected in EDTA tubes from 40 randomly selected patients treated for mCRPC at the Royal Marsden Hospital between in 2016 for FBC and immunophenotyping by flow cytometry. The NLR is a quotient of the neutrophil count and lymphocyte count. Whole blood samples were processed by red cell lysis then stained for CD45, CD11b, CD15, HLA-DR, and CD14. We gated for singlets, then mononuclear cells. PMN-MDSCs and M-MDSCs were defined as CD45+CD11b+CD15+HLA-DR-CD14- and CD45+CD11b+CD15-HLA-DR-CD14+, respectively, and expressed as a fraction of the CD45+ population. **Statistics:** Patient demographics, NLR, and PMN- and M-MDSC frequency are presented descriptively. Pearson correlations were performed for associations between NLR and PMN MDSCs and NLR and M-MDSCs.
Results: We analysed samples from 40 mCRPC patients (age 67.0 [range 51-89]) who were ECOG performance status 0-1. The proportion of PMN-MDSCs as a fraction of total CD45+ cells was 0.3% (interquartile range [IQR]: 0.17-0.57%) and the proportion of M-MDSCs as a fraction of total CD45+ cells was 0.18% (IQR: 0.17-0.44%) with the median ratio of PMN-MDSC to M-MDSCs being 2.1 (IQR: 1.7-11.0). We showed that NLR was positively associated with the frequency of PMN-MDSC (r = 0.56, p<0.001) and M-MDSCs (r = 0.5, p=0.0012) in the peripheral circulation. We showed that neutrophil count was positively associated with the frequency of PMN-MDSC (r = 0.36, p=0.02) and M-MDSCs (r = 0.36, p=0.15). Lymphocyte count was inversely proportional to the frequency of PMN-MDSCs (r = -0.35, p=0.03) and M-MDSCs (r = -0.41, p=0.008).
Conclusions: We describe the frequency of PMN and M-MDSCs in the peripheral blood of patients with mCRPC, and demonstrate relationships between peripheral blood neutrophil, lymphocyte count and NLR with MDSC frequency in patients with mCRPC.

IMM-08

EFFECT OF INFLIXIMAB TREATMENT ON THE DISTRIBUTION OF NK, MAIT, INKT AND TCR γδ CELLS IN CHILDREN WITH CROHN'S DISEASE

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Introduction: Tumor necrosis factor alfa (TNF-alfa) is targeted by inhibitor such as infliximab (IFX) which neutralizes this cytokine. Although there are a few studies showing that innate lymphocytes are very sensitive to therapeutic doses of glucocorticoids, the effect of infliximab on the innate lymphoid cells (ILC) and unconventional T cells in children with Crohn's disease (CD) remains unknown. Hence, the aim of this study was to investigate the effect of infliximab treatment on the distribution of circulating type 1 ILC, MAIT, iNKT, and TCR *γδ* in children with CD.
Methods: Nine pediatric patients with CD (before and after IFX treatment (after 2 doses of IFX and after 1 year therapy) were included. Aged-matched healthy children served as a control group. The distribution of circulating type 1 ILC ($CD3^-/CD56^+/CD16^+$) and unconventional T cells, i.e. MAIT (TCR $V\alpha7.2^+/CD161^+$), iNKT ($CD3^+$/TCR $V\alpha24\text{-}J\alpha18^+$) and TCR *γδ* ($CD3^+$/TCR *γδ* $^+$) were evaluated in the peripheral blood by flow cytometry (BD FACSCanto II).
Results: We showed the decreased percentage of NK, MAIT and iNKT cells in the peripheral blood of children with CD before starting IFX treatment compared to the control group (*p<0,05*). The IFX induction therapy excacerbated the decline of circulating NK population along with the increase in TCR *γδ* cells. Unfortunately, this distribution profile was not observed after one year IFX therapy.
Conclusions:
Our results demonstrated that children with CD before starting biological therapy due to ineffective conventional treatment were characterized by a reduced percentage of peripheral NK cells, MAIT and iNKT cells. Unfortunately, one year therapy with IFX did not change the distribution of these cells.

IMM-09

LYMPHOCYTE AND CYTOKINE PROFILES IN PATIENTS WITH TYPE 1 DIABETES AND HASHIMOTO'S DISEASE

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Introduction: Autoimmune diseases constitute a very numerous group of disorders. They are very often accompanied by chronic inflammation regulated by cytokines. Both type 1 diabetes and Hashimoto's disease are classified as autoimmune diseases. The objectives of the present study were: to determine the proportion of specific T lymphocyte subpopulations (T CD4+, T CD8+) in the peripheral blood of patients with type 1 diabetes, Hashimoto's disease and patients with both conditions, and evaluation of the profile of selected cytokines specific for Th1 and Th2 helper lymphocytes in the peripheral blood serum of patients depending on the disease entity diagnosed.
Methods: The samples were acquired from a group of 50 patients, 21 boys and 28 girls (1 patient with no gender indication), with median age being stated as 14.5 years old. The youngest patient participating in the study was 3.92 years old and the oldest was 18 years old. The immunofenotyping of peripheral blood leukocytes and the assessment of cytokine concentrations in blood serum were performed by flow cytometry [CyFlow Cube 8, CyFlow Space, Sysmex].

Results: It was found that the highest level of lymphocytes (41.635%) was in patients with type 1 diabetes (similar to the percentage of lymphocytes in patients with Hashimoto's disease – 39.770%), while patients with both type 1 diabetes and Hashimoto's disease had the lowest percentage of lymphocytes (30.260%). The predominance of T lymphocytes over B lymphocytes was confirmed; within the T lymphocytes, the predominance of CD4+ lymphocytes over CD8+ lymphocytes was observed. In patients with diabetes, the percentage of CD8+ T lymphocytes was slightly lower than in the other groups (20.260%). Patients with type 1 diabetes and Hashimoto's disease showed the lowest percentage of B-lymphocytes and T-lymphocytes among the whole study group. The highest CD4+/CD8+ ratio (6.94) was found in patients with type 1 diabetes, the lowest in patients with both Hashimoto's disease and type 1 diabetes (1.103). Comparing medians for the CD4+/CD8+ ratio the highest value is observed for type 1 diabetes (2.770), the lowest for Hashimoto's disease (2.158).
Conclusions: The highest immune response is shown in people with type 1 diabetes, while the lowest in those with diabetes mixed with Hashimoto's disease. The study showed sexual dimorphism in immunological components visible in lymphocyte subpopulations. The results of the study indicate mixed Th1/Th2 pathomechanism in type 1 diabetes and/or Hashimoto disease.

OTHER (OTH)

OTH-01

EVALUATION OF TRACEABILITY IN VARIOUS TB&NK WORKFLOWS TO ASSESS STAGES OF ERRORS PRONENESS AND AUDITABILITY

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Introduction: Specimens and reagents traceability is integral to Good Laboratory Practice (GLP). Current staffing challenges and increased workloads demand high traceability. Various flow cytometry workflows employ specimens and reagent barcodes and audit trails throughout the process: (1) manual methods with visual confirmation or logs; (2) semi-automation enables increased auto-traceability; and (3) fully integrated automation systems have high auto-traceability. Objective methods for traceability evaluation can inform automation selections to enhance GLP.
Methods: Six Sigma attributes methodology was utilized to score TB&NK workflow stages of (a) preparation (b) middleware involvement, and (c) acquisition/analysis. These were further sub-staged for specimen and reagents tracking with a score of 0 with visual confirmation, 1 with manual entry into software or paper log, and 2 for barcode enabled auto-entry. Each stage was identified from manufacturer product-specific Instructions for Use, then classified as error prone and/or auditable.
Results: For all applicable workflow traceability stages: (1) Manual method had a mean traceability score of 0.76/2.00, with auditability of 41.7%, and 75.0% of stages being error prone. (2) Semi-automated mean traceability score was 1.06/2.00, with auditability of 62.9%, and 62.9% of stages being error prone (3) Fully integrated mean traceability score was 1.97/2.00, with auditability of 80.6%, and 6.5% of stages being error prone.
Conclusion: Fully integrated automation systems (physical and data integrated) running TB&NK testing provide the best scores using this objective method. This method is simple to apply when evaluating next generation systems to address staffing challenges and increased workloads.

OTH-02

INTERFERENCE EVALUATION OF THE BD® STEM CELL ENUMERATION KIT ON THE BD FACSLYRIC™ FLOW CYTOMETER

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Introduction: The BD® Stem Cell Enumeration Kit is a single-tube in vitro diagnostic assay, intended for enumeration of viable dual-positive CD45+/CD34+ hematopoietic stem cell populations to determine viable CD34+ absolute counts (cells/µL) and the percentages of viable CD45+/CD34+ hematopoietic stem cells. The BD® Stem Cell Enumeration (SCE) Kit was evaluated using fresh cord blood, fresh bone marrow and fresh leukapheresis in the presence of endogenous and exogenous interfering substances on the BD FACSLyric™ Flow Cytometer using the BD FACSuite™ Clinical Application with the BD® Stem Cell Enumeration module for acquisition and analysis. The interferents evaluated included substances likely to be present in patient specimens and were evaluated according to CLSI EP07*, 3rd Edition.* The concentrations tested were based on three times the maximum therapeutic dose observed in patients or the highest expected concentrations per CLSI EP07 Supplement (EP37)*, 1st Edition*.
Methods: The substances evaluated in this study included albumin, bilirubin, cyclophosphamide, hemoglobin, doxorubicin, GCSF, intralipid and paclitaxel. For each of the specimen types, 15 replicates were stained using the BD® SCE Kit for each interferent condition. A total of nine specimens comprising three leukapheresis, two bone marrows, and four cord bloods were used to complete the evaluation. A paired-difference analysis was used to evaluate the Control (no interferents) versus the Test (with interferents) conditions.
Results: Leukapheresis, bone marrow and cord blood exhibited a maximum mean bias of -3.2% (-10.5, 4.0), -4.7% (-11.6, 2.1) and -1.2% (-2,86, 0.46), respectively, for viable CD34+ absolute count in the presence of the highest concentrations of interferents evaluated. In the presence of interfering factors, the maximum %CV for CD34+ absolute count in leukapheresis, bone marrow and cord blood specimens were 12.2%, 11.4% and 11.6%, respectively.
Conclusions: There was no detectable interference to the enumeration of viable CD34+ absolute counts from the interfering substances tested at their highest concentrations.
The BD FACSLyric™ Flow Cytometer is a Class 1 Laser Product. In the EU., the BD FACSLyric™ Flow Cytometer with the BD FACSuite™ Clinical and BD FACSuite™ Applications is an in vitro diagnostic medical device bearing a CE mark.
The BD® Stem Cell Enumeration Kit and BD® Stem Cell Controls are in vitro diagnostic medical devices bearing a CE mark and are CE certified by BSI Group the Netherlands B.V. (Notified Body Number = 2797).