ESCCA
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for Clinical Cell Analysis

ESCCA 2016

A "Festival" of Cell Analysis

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ABSTRACT BOOK

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OPENING SESSION LECTURE

Overview of the Education Programme and the ESCCA Education Strategy for the Future

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One of the major pillars of the ESCCA Strategic Plan for year 2020 is Education. ESCCA Education Programme includes several initiatives for ESCCA members. The International and Local Schools on Cytometry, held in Valencia, Urbino and Saint Etienne provide excellent oportunities for the interactions of students with experts, and cover basic and especialized issues of cytometry through theoretical and practical sessions. ESCCA is also developing two e-learning tools for self-assessment in cytometry: a) the TYSCCA project (Test Yourself in Clinical Cell Analysis) is a repository of quizzes which can be used to test and improve oneself in cytometry; b) the ESCCABase project includes a repository of certified FCS data from clinical cases and basic experiments that can be freely downloaded and re-analyzed by the cytometric community using ESCCA Viewer, a non-commercial software developed purposely by ESCCA to work on ESCCABase files. The strategy of the Education and Accreditation Committee will focus in three main objectives: a) To ensure that ESCCA educational initiatives activities will be officially acknowledged with CME credits from european and/or local authorities, as a way of attracting young clinical professionals into ESCCA meetings and courses; b) To establish the European Cytometry Certificate for ESCCA members, with two levels of certification, namely 'Cytometry Operator', after evaluation of technical skills and basic knowledge by means of a written exam, and 'Cytometry Specialist', after evaluation of skills and knowledge in applied fields of cytometry (Biological, Biotechnological or Clinical applications) by means of a written exam and the revision of the CV (minimum of three-year experience in cytometry) submitted by the applicant. The contents of the different basic and specialized courses of ESCCA Schools, as well as TYSCCA and ESCCABAse will address the items required pass successfully the exams for certification; c) To attain the validation of the ESCCA Cytometry Certificate (Cytometry Operator) by the appropriate european medical authorities. Finally, regarding the ESCCA policy on the dissemination of and collaboration with educational activities, the Education and Accreditation Committee has published a series of basic criteria for evaluation of all courses wishing to be announced in ESCCA website or requesting ESCCA auspices and collaboration. ESCCQ may aprove financial sponsorship to courses organized by cytometry societies or official institutions of low-income countries, as well as to courses organized by non-profit insitution in countries without cytometry society. ESCCA support will be applied to student fellowships or to travel of ESCCA members invited as teachers.

PLENARY SESSION 1: KEYNOTE LECTURES

PLE-01-01

Remodeling the clinical flow lab in response to new technologies and treatments

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New cancer therapies are moving from cytotoxic drugs with generalized toxicity to those that specifically target tumor cells. Antibody therapies have become common in several leukemias/lymphomas but new antibody therapies are being introduced. One new direction, CAR T-cell therapy, utilizes the patient's immune system and gene therapy to activate T-cells to attack and kill tumor cells. This new type of therapy is being utilized against leukemia/lymphoma and solid tumors. Synthetic inhibitors of specific molecules involved in tumor growth and progression are also being widely tested. The reduced toxicity and good response rates mean these therapies are currently important in the academic setting and in the future will impact other clinical institutions. These new therapies provide new opportunities for growth in clinical flow cytometry testing but also present new challenges. The masking of CD20 by rituximab therapy is a well-known phenomenon but new anti-body based therapies, e.g. anti-CD38 in myeloma, are constantly being introduced resulting in apparent negativity for key antigens used in identifying disease. Furthermore, relapsed disease post CAR T-cell therapy can also appear negative for the targeted antigen. Therapy utilizing inhibitors of signaling pathways can affect multiple cell processes, resulting in modified expression of multiple proteins and an altered immunophenotype. The resulting changes in immunophenotype can make detection of residual disease difficult unless the panels and method of analysis are sufficiently robust and flexible to adjust to unexpected changes. These novel therapies are also providing new opportunities for clinical flow cytometry testing. Targeted antigens must be expressed on the cell surface, making flow cytometry the optimal method of screening for antibody and CAR T-cell therapies. Flow cytometry is also optimal for quantitation of CAR T-cell levels. In addition, multi-parametric flow cytometry can detect complex simultaneous changes in numerous antigens expressed by cells of different lineages, making it useful in identifying the full spectrum of therapeutic effect. As we move into the age of individualized medicine, the flow cytometry laboratory must be prepared to constantly adapt to unexpected changes in immunophenotype in residual/relapsed disease while at the same time being ready to grasp the new opportunities these novel therapies provide.

PLE-01-02

Remodeling of the stromal niche in haematological malignancy

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Acute myeloid leukemia (AML) is a hematologic malignancy, arising within the bone marrow, which is characterized by the uncontrolled proliferation of leukemic blasts, often in association with a disruption of normal hematopoiesis. Like their normal counterparts, AML cells depend upon both cell-intrinsic and -extrinsic regulatory signals generated by their surrounding microenvironment, for their survival and proliferation.
AML has long been considered a hematopoietic-cell autonomous disorder in which disease initiation and progression is driven by hematopoietic cell intrinsic genetic events. Recent experimental findings in diverse model systems have challenged this view, implicating different stromal cells of the bone marrow in disease pathogenesis. Thus it is now accepted that leukemic hematopoiesis can turn the BM niche into a "leukemic niche" which promotes leukemic stem cell (LSC) function and impairs the maintenance of normal HSC. However, much remains to be understood about how different leukemic cells impacts the BM microenvironment and, in turn, how changes in the activity of specific BM niche cells contribute to AML pathogenesis. This talk will highlight some of the current understanding of the alterations of BM niche components and how the dialogue between leukemic and stromal cells participated in leukemogenesis.

PLENARY SESSION 3: ESCCA-ISAC JOINT SYMPOSIUM

PLE-03-01

Highly multiplexed analysis of tissues by imaging mass cytometry

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Cancer is a tissue disease. Heterogeneous cancer cells and normal stromal and immune cells form a dynamic ecosystem that evolves to support tumor expansion and ultimately tumor spread. The complexity of this dynamic system is the main obstacle in our attempts to treat and heal the disease. The study of the tumor ecosystem and its cell-to-cell communications is thus essential to enable an understanding of tumor biology, to define new biomarkers to improve patient care, and ultimately to identify new therapeutic routs and targets.
To study and understand the workings of the tumor ecosystem, highly multiplexed image information of tumor tissues is essential. Such multiplexed images will reveal which cell types are present in a tumor, their functional state, and which cell-cell interactions are present. To enable multiplexed tissue imaging, we developed imaging mass cytometry (IMC). IMC is a novel imaging modality that uses metal isotopes of defined mass as reporters and currently allows to visualize over 50 antibodies simultaneously on tissues with subcellular resolution. In the near future we expect that over 100 markers can be visualized.
We applied IMC for the analysis of hundreds of breast cancer samples in a quantitative manner. To extract biological meaningful data and potential biomarkers from this dataset, we developed a novel computational pipeline geared for the interactive and automated analysis of large scale, highly multiplexed tissues image datasets. Our analysis reveals a surprising level of inter and intra-tumor heterogeneity and identify new diversity within known human breast cancer subtypes as well as a variety of stromal cell types that interact with them. Furthermore, we identified cell-cell interaction motifs in the tumor microenvironment correlating with clinical outcomes of the analyzed patients.
In summary, our results show that IMC provides targeted, high-dimensional analysis of cell type, cell state and cell-to-cell interactions within the TME at subcellular resolution. Spatial relationships of complex cell states of cellular assemblies can be used as biomarkers. We envision that IMC will enable a systems biology approach to understand and diagnose disease and to guide treatment.

PLE-03-02

Instrument development in flow cytometry: ways to combine full spectral with fluorescence lifetime

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Introduction: The international flow cytometry community consistently uses fluorescence activated cell sorters and counters to solve a wide range of biomedical, biophysical, and biomaterial engineering problems. The variety of cytometry instruments is quite vast and ranges from imaging cytometers, simple counters, multi-color cell sorters, to full spectral cytometry instruments. The variety of systems are continuously modified in ways that improve the optical measurements as well as to be able to capture different photophysical traits of fluorescent molecules. For example, fluorescence decay kinetic measurements are now possible with time-resolved flow cytometers, which measure fluorescence lifetimes for cell counting and sorting. In the past several years our laboratory has made strides toward adapting lifetime technologies onto existing commercial systems to effectively make fluorescence lifetime measurements simple and achievable for a range of cytometric architectures. Additionally, our laboratory has interest in combining fluorescence lifetime measurements with full spectral data while maintaining standard cytometric throughputs.
Methods: In this contribution we describe the various ways in which cytometry hardware (i.e. detectors, lasers, optics, cameras, data acquisition systems, etc) can be modified to measure the fluorescence lifetime, full spectral data, or the combination thereof. We focus mainly on measuring the fluorescence lifetime, however provide insight on combining spectral data with decay kinetic measurements.
Results: Commercial cytometers can be modified to measure fluorescence decay kinetics in ways that include frequency-domain electro-optic systems as well as hybrid time-domain systems. There is potential to adapt time-resolved measurements onto existing full spectral systems, depending on the detector architecture and signal processing steps. A wide range of cell applications and biomedical assays might benefit from time-resolved and full spectral data capture including measurements of intracellular Förster resonance energy transfer, metabolic mapping, discrimination of autofluorescence species, multiplexing, and barcoding.

Conclusions: It is non-trivial to capture heterogeneous time-resolved information from several different excitable molecules when excitation is observed from single cells and particles in fluidic states. Therefore we discusses and summarize new techniques that might expand how multi-exponential fluorescence decay and/or full spectral flow cytometry can add to the information content for high throughput cell counting and sorting.

PLENARY SESSION 4: STANDARDISATION

PLE-04-01

EuroFlow

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Background: Immunophenotyping of normal, reactive and malignant leukocytes by flow cytometry (FCM) is currently used to support the diagnosis, the prognostic classification and the evaluation of effectiveness of treatment of most hematological malignancies. As a clinical test, standardization of FCM immunophenotyping becomes a must.
Key objectives: The EuroFlow Consortium decided for: 1, full standardization of all technical procedures; 2, design of new software tools for facilitating easy and reproducible processing of FCM data, including pattern recognition based on n-dimensional spaces; 3, design of 8-12 color antibody panels for the diagnosis, classification, and monitoring of hematological malignancies; and 4, creation of reference data bases.
Results: The Consortium designed the EuroFlow diagnostic algorithm with entries defined by clinical and laboratory parameters and further consisting of validated EuroFlow 8-color antibody panels for immunophenotyping of hematological malignancies: single-tube screening panels for diagnosis and multi-tube classification panels. The panels were constructed in 2-7 sequential design-evaluation-redesign rounds, using novel Infinicyt software tools for multivariate data analysis. Two groups of markers are combined in each 8-color tube: i) backbone markers to identify distinct cell populations in a sample, and ii) markers for characterization of specific cell populations. In multi-tube panels, the backbone markers were optimally placed at the same fluorochrome position in every tube, to provide identical multidimensional localization of the target cell population(s). The characterization markers were positioned according to the diagnostic utility of the combined markers. Each proposed antibody combination was tested against reference databases of normal and malignant cells from healthy subjects and WHO-based disease entities, respectively. The EuroFlow studies resulted in validated and flexible 8-color antibody panels for multidimensional identification and characterization of normal and aberrant cells, optimally suited for immunophenotypic screening and classification of hematological malignancies. Ongoing projects within the EuroFlow Consortium are now mainly focused on: 1) the construction of reference data bases for the 8-color EuroFlow antibody panels; 2) the design of minimal residual disease tubes for monitoring the effectiveness of therapy; and 3) design of screening and diagnosis tubes for patients suspected to have an immunodeficiency.
Future perspectives: The “all-in-one” EuroFlow concept and the technical achievements of EuroFlow will facilitate standardization of diagnostic FCM and the multicolor antibody tubes/panels in combination with reference data bases will provide more reliable and reproducible diagnostics in immunology and hematology.

PLE-04-02

Harmonemia, an international experience of harmonization in flow cytometry

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Introduction: The world of flow cytometry is multifaceted and highly flexible. This makes it sometimes difficult to compare results obtained from different instruments or softwares. It is also important to be able to harmonize different instruments in a given platform or among a working group.
Methods: The Harmonemia project has been designed to test the feasibility of a simple and robust way of setting the parameters of a number of instruments and compare the data obtained. The voltage of photomultipliers (PMT) was set in a reference laboratory, using lyzed unstained normal blood and obtaining at least 80% of unstained cells above channel 1. Fluorescent beads were then run in this same setting, using the PMT established with unstained blood. Targets values for this batch of beads, as well as aliquotes of the latter were then distributed to 17 cytometry platforms, housing a total of 23 instruments from both major manufacturers. The precision of PMT setting, validity of targeting unstained cells and staining of peripheral blood and bone marrow samples with a specific antibody panel were then evaluated.

Results: High reproducibility was obtained for all parameters studied and for the 23 instruments. The strategy of using unstained lyzed blood to adjust/test PMT settings proved both very simple and robust. Highly superimposable stainings were obtained for the major subsets of mature leukocytes in peripheral blood and bone marrow, whatever the instrument used. A greater interindividual variability was identified among bone marrow samples, yet superimposition or merge of listmode files allows to provide a global representation of normal bone marrow.
Conclusions: The Harmonemia strategy for flow cytometry harmonization appears to be robust and readily applicable in any laboratory. The use of beads with fluorescence targets facilitates the setting of PMT in the same platform or among a group of users. An atlas of normal bone marrow analyzed according to Harmonemia recommendations is in preparation, which will be posted on the website of the European LeukmiaNet. Such a tool should prove very valuable for the flow cytometric analysis of myelodysplasias and minimal residual disease in hematological malignancies.

PLE-04-03

Standardisation: the ESCCA approach
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Introduction: It is critical that clinical assays meet an appropriate standard but there is a recognised need for flexibility due to resource, platform and reagent availability. ESCCA harmonisation projects aim to identify i) the minimum requirements for a clinical diagnostic assay; ii) additional recommended markers for further harmonisation in better-resourced laboratories; iii) performance criteria for those reagents that are platform- and supplier-independent.
Methods: The ESCCA harmonisation group identifies relevant topics by survey of the members to determine the most pressing requirements for harmonisation and validation. Based on the experience of many different consensus project, the proposed approach is:
1) Develop laboratory and clinical working groups for specific diagnostic topics
2) Survey members to identify consensus on required (for all resource settings) vs. recommended reagents.
3) Identify positive and negative control populations (preferably in normal blood) with a minimum relative signal to specify appropriate antibodies
4) Define a consensus approach and test this retrospectively if possible. Confirm the consensus after consultation with members and publish.
5) Test the consensus approach prospectively and identify diagnostic challenges.
In collaboration with the ICCS Quality and Standardization committee, the ESCCA harmonisation groups will further develop and define expectations for optimal instrument settings, reagent selection and specimen preparation.
Results: The ESCCA harmonisation group has successfully completed two consensus projects so far:
In collaboration with ICCS: “The Flow Cytometric Detection of Minimal Residual Disease”. January 2016, Cytometry B Special Issue. Volume 90 pages 1-100.
In collaboration with ERIC: “Reproducible diagnosis of Chronic Lymphocytic Leukemia by flow cytometry”. The consensus document was opened for consultation by ESCCA members and is now in review for publication.
A survey to identify the next harmonisation topics was distributed to ESCC/ICCS/ERIC members. The survey closed on July 31 and the top three topics were “Chronic Myelomonocytic Leukemia Diagnosis”, “Mycosis Fungoides/Sézary Syndrome Diagnosis” and “Hairy Cell Leukemia & Variants: Diagnosis and Monitoring”. Further progress on the evolution of these projects will be reported in Edinburgh.
Conclusions: ESCCA is working closely with clinical and scientific partners to identify solutions for clinical issues that can be used by all of the members. In particular, there is strong partnership with the ICCS quality and standards committee to facilitate their goal of optimizing fundamental flow cytometric testing components. Members are strongly encouraged to join in the projects because they provide a great opportunity for mutually beneficial education and dissemination of good practice.

PLENARY SESSION 5: REGENERATIVE MEDICINE

PLE-05-01

Stem cells for joint repair and arthritis

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Symptomatic full-thickness lesions of the articular cartilage of the knee joint require surgical treatment for symptom relief and to prevent evolution towards secondary post-traumatic osteoarthritis (OA). Currently, autologous chondrocyte implantation (ACI) represents gold standard of cell therapy for cartilage repair. However, during culture expansion articular chondrocytes rapidly undergo dedifferentiation in vitro, losing their capacity to form cartilage when transplanted in vivo. The use of mesenchymal stem cells (MSCs) as chondrocyte substitutes in an ACI-equivalent procedure is intensely pursued. MSCs are easily accessible from bone marrow and connective tissues such as the synovium, easy to expand in culture, and they have ability to form cartilage and bone. In addition, MSCs appear to be immune privileged, at least under specific conditions. These properties would allow generation of MSC preparations ready for allogeneic use, thus circumventing the limitations and patient-to-patient variability of autologous cell protocols. Pharmacological studies are required to determine identity, purity and potency of the MSC products. Clinical studies are needed to compare MSCs with articular chondrocytes to demonstrate their clinical and structural non-inferiority in joint surface repair. Intra-articular delivery of MSCs in patients with OA is also being pursued, and some promising data are emerging from clinical studies.
Another approach to cartilage repair is the activation of intrinsic regenerative mechanisms by local implantation into the defect of a functionalised biomaterial or administration of medications that target the stem cells naturally present in the joint.
There is therefore potential for multiple therapeutic approaches to joint surface repair. It is anticipated that the type of intervention will be dependent on clinical indication and factors such as size of the lesion, status of the surrounding cartilage and other joint tissues, stage of OA. Looking forward, regenerative interventions will have to be tailored and patient-specific, thus emphasising the need for patient stratification towards personalised and precision medicine.

PLE-05-02

Measuring cellular function and identity for safety and efficacy of advanced cellular therapeutics

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Cellular therapeutics are a growing area of medicine, particularly therapies aimed at regenerating diseased tissue, as well as immune therapies for infection and cancer. SNBTS is one of the largest centres for development of cellular therapies in the UK, and has developed and manufactured stem cell and immune therapies for nearly 20 years. Flow cytometry is arguably the most important tool in the development of advanced cellular therapeutics. Cellular growth and death, states of differentiation as well as viability are essential data to be collected and documented. This requires robust and validated flow cytometric analysis. Often ruler-materials need to be generated and validated, in order to provide meaningful comparison materials.
In this presentation, I will discuss the design and validation of cellular release assays: how to take into account variation in raw donor materials and cell processing efficiency, yet build a robust set of release criteria. I will also discuss the building of flow cytometric assays to replace in vitro cellular functional assays to allow “on the day” release of cellular products. This will be illustrated through the development of 2 complex advanced therapeutics, with very different analytical requirements - Virus specific T cells for treatment of virally-driven tumours, and autologous macrophages for the treatment of liver disease.

PLENARY SESSION 6: APOPTOSIS FROM BENCH TO BEDSIDE

PLE-06-01

Apoptosis and Inflammation

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Inflammation is involved, in some form or another, in virtually all diseases. Inflammatory leukocytes, especially granulocytes and macrophages, are key cells of host defence. However, excessive leukocyte recruitment, dysregulated leukocyte function and/or failed clearance of effete cells will result in tissue damage associated with inflammatory diseases. Furthermore, the resolution of inflammation, that occurs when inflammation abates, is an important, active and highly regulated phenomenon. My group has focused on key processes that occur during inflammation resolution; namely apoptosis (programmed cells death) and efferocytosis (non-phlogistic phagocytosis of apoptotic cells by phagocytes especially macrophages). I will present evidence on how these processes can be manipulated pharmacologically to enhance the resolution of inflammation and how such studies could lead to the identification of novel therapeutic targets for the treatment of both acute and chronic inflammatory diseases. I will focus upon how flow cytometric analysis and flow cytometric cell sorting have helped identify important pathways involved in inflammation resolution. We have used flow cytometry to successfully measure leukocyte function, apoptosis and macrophage efferocytosis. In addition, we have developed a cell sorting technique to isolate highly pure non-perturbed neutrophils for further study. Furthermore, flow cytometry has helped in the characterization and phenotypic analysis of inflammatory leukocytes from patients with inflammatory diseases. Recent advances in imaging flow cytometry and mass cytometry will undoubtedly provide further insights in the leukocyte biology research field. An update on how manipulation of inflammation resolution by influencing apoptosis is being translated into the clinic will be provided.

PLE-06-02

Apoptosis and Leukaemia: from bench to bedside

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The pathophysiology of chronic lymphocytic leukaemia (CLL) is characterized by an accumulation of CLL cells due to their increased proliferation, due to aberrant signaling through the B-cell receptor (BCR), and their decreased apoptosis, due to the ubiquitous expression of anti-apoptotic machinery typified by the over-expression of Bcl-2. The major advances in treating CLL over the last 5 years have resulted from a better understanding of this dual pathogenesis and the specific targeting of both BCR signaling, mainly through the irreversible inhibition of Bruton tyrosine kinase (Btk), and of apoptosis, through the inhibition of Bcl-2 by venetoclax. CLL cells are in fact primed for apoptosis but this is held in check by the Bcl-2 over-expression. One of the most notable features of venetoclax treatment is the propensity to result in tumour lysis syndrome that has in the early experience been extremely rapid and life-threatening. This has led to a gradual escalation of the dose of venetoclax over the first week of treatment to ameliorate this complication and largely such an approach has been very effective.
A key difference between the BCR-antagonists, such as ibrutinib, and venetoclax is that although ibrutinib is extremely effective it doesn't lead to the eradication of detectable minimal residual disease whereas venetoclax does do so in a significant proportion of patients. In addition, as expected, the early data indicates that venetoclax synergizes with both monoclonal antibodies and chemo-immunotherapy to yield even higher rates of MRD negativity than with monotherapy. Trials are currently on-going examining the combination of ibrutinib with venetoclax as from a pathophysiological perspective this combination is very compelling.
The laboratory assessment of Bcl-2 expression is useful to identify those patients who are most likely to benefit from venetoclax but in reality the expression is universally high in CLL. However the level of the CLL counts, bulk of disease and renal function are all used to predict the likelihood of significant tumour lysis. MRD assessment is critical to defining the duration of venetoclax and is currently being studied in clinic.
In summary the promise of anti-apoptotic therapy in CLL is now, after many decades, being realized with the promise that specific targeted therapy will lead to shorter duration of effective, targeted treatment.

PLE-07-01

Association of basophil immunophenotypic features of promyelocytic leukemia cells and life-threatening hemorrhage at diagnosis: a multicenter study on 118 patients

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Introduction: Occurrence of severe hemorrhage in acute promyelocytic leukemia (APL) at diagnosis or during induction therapy is a major focus of research in APL. We investigated the potential association between the phenotypic profiles of bone marrow (BM) APL leukemic cells and the degree of bleeding diathesis at diagnosis.
Methods: 118 BM samples from newly-diagnosed APL patients were characterized by multiparameter flow cytometry using the EuroFlow approach. Bleeding diathesis (e.g. absent vs. mild and severe) and its relationship with the immunophenotypic features of blasts at diagnosis was studied in 102 cases.
Results: A higher peripheral blood (PB) but not BM leukemic infiltration was detected in APL with (any) bleeding symptoms vs. those without bleeding manifestations (45% vs. 16% PB blasts; p=0.01), although without differences between cases with mild and severe hemorrhage.
Phenotypically, BM leukemic cells systematically depicted: $CD117^{+/het}$, $CyMPO^{+/++}$, $CD13^{+/++}$, $CD15^{-/dim}$, CD64, $CD33^{+/hi}$, $CD71^{dim}$, CD38, and $CD123^{+/hi}$ expression, while lacking HLA-DR and other lineage-associated markers. Albeit at lower frequencies, other phenotypes involved the partial expression of the basophil-related markers CD203c and CD22 (43% and 14% of cases), followed by $CD4^{lo}$ (35%), CD34 (28%), CD35 (27%), CD7 and CD56 (20%).
Noteworthy, detection of basophil-lineage features on BM blasts showed the strongest association with the extent of hemorrhage; an increasing frequency of cases with BM $CD203c^+$ and/or $CD22^+$ blasts was noted from APL without bleeding to APL with mild and severe hemorrhage (15% to 45% and 80% of cases; p<0.001). Furthermore, also the percentage of BM $CD203c^+$ blasts increased (2% to 2.5% and 10%; p<0.001), with significant differences between mild and severe bleeding diathesis (p=0.003).
Based on the percentage of BM $CD203c^+$ cells by flow cytometry and the blast infiltration in PB smears, a higher frequency of PB $CD203c^+$ leukemic cells was extrapolated in patients with bleeding symptoms. Proportionately, an increasing average of PB $CD203c^+$ leukemic cells was estimated in APL without bleeding vs. those with mild and severe traits (0.2% vs. 0.8 and 4%; p<0.001); noticeable, higher percentages of PB $CD203c^+$ leukemic cells were found in cases with severe bleeding (p=0.03), together with increasing absolute numbers of these cells (0.01 vs. 0.06 and 0.1 $x10^9$/L; p=0.004).
Finally, detection of >1.6% $CD203c^+$ BM leukemic cells was associated to the highest sensitivity and specificity for severe bleeding (p<0.001).
Conclusion: The detection of basophil differentiation features by BM APL blasts may be useful to predict an enhanced risk for more severe bleeding manifestations at diagnosis.

PLE-07-02

Integrated single cell network profiling data of ERK signaling and mutations of SF3B1 gene refine prognosis in chronic lymphocytic leukemia

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Introduction: Extracellular signal-regulated kinase (ERK) is a major pathway downstream of the B-cell receptor (BCR) and its activation is a significant, independent predictor of shorter clinical progression in chronic lymphocytic leukemia (CLL). Recently identified driver genetic lesions, most likely favored by BCR signals, have reshaped the genetic landscape and added a further level of complexity in CLL. Despite the established driving role of BCR signaling and gene mutations in pathobiology and clinical behavior of CLL, little is known about the clinical influence of integrated BCR responses and genetic alterations. In this study, we investigated the clinical impact of integrated BCR response of ERK and gene mutations in CLL.
Methods: Peripheral blood cell samples at diagnosis from 152 CLL patients were analyzed in this study. ERK phosphorylation was analyzed using Single Cell Network Profiling (SCNP), a flow cytometry-based assay that allows signaling analysis at the single cell level. *NOTCH1*, *SF3B1*, *TP53*, *MYD88*, and *BIRC3* gene mutations were analyzed by direct DNA sequencing. Univariate and multivariate models for time to first treatment (TTFT) were generated using Cox proportional hazards regression. TTFT curves estimated using the Kaplan-Meier method for the respective groups of patients were compared using the log-rank test.
Results: BCR stimulation with anti-IgM induced a signaling response of ERK (anti-IgM→p-ERK) that was significantly higher in UM subset (P=0.0020), in CD38-positive CLL (P=0.0059), in treated patients (P=0.0003), and in mutated-*SF3B1* cells (P=0.0098). Univariate analysis identified increased anti-IgM→p-ERK (P=0.001), UM-*IGHV* (P<0.0001), positive CD38 (P=0.001), mutated *SF3B1* (P<0.0001), as significant, independent predictors of shorter TTFT. In multivariate analysis, only increased anti-IgM→p-ERK (P=0.03) and *SF3B1* mutation (P=0.0001) were independent significant parameters of prognosis. Integrating anti-IgM→p-ERK data and *SF3B1* mutations stratified patients in three independent prognostic categories (P<0.0001) and identified an intermediate-risk group that included patients with low p-ERK and mutated *SF3B1* or high p-ERK and wild-type *SF3B1*. Consistent with the results in the whole patient set, integrating anti-IgM→p-ERK and *SF3B1* mutation in an unselected group of Binet stage A patients (n=110) identified an intermediate-risk group including patients with low p-ERK and mutated *SF3B1* or high p-ERK and wild-type *SF3B1* (P=0.0002).
Conclusions: These data reveal that integrated dynamic ERK signaling and *SF3B1* mutations independently predict disease progression risk in CLL and identify a novel risk-group of patients, thus providing complementary prognostic information and suggesting a functional synergy between BCR-induced ERK signaling and altered *SF3B1*.

PLE-07-03

Comparison of B-lineage precursor cell markers expression in regenerating vs. normal bone marrow

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Introduction: B-lineage precursor cells (BCP) constitute a normal subpopulation of immature cells in bone marrow. There are four main maturational stages of BCP: pro-B-cells, pre-B-I-cells, pre-B-II-cells and immature/transitional B-cells differing with immunophenotypic features. The purpose of the study was to compare the immunophenotypes of different types of BCP between normal and regenerating bone marrow by assessing the profile of expression of cIgM, TdT, CD22, CD27, CD19, CD34, CD38, CD10, CD20 and CD45 antigens.
Methods: Regenerating, minimal residual disease negative bone marrow samples were collected from 10 children (median age: 2,8 years) with acute lymphoblastic leukemia at week 12 of chemotherapy. The control group comprised 9 patients (median age: 3.5 years) with nonmalignant hematologic conditions, mainly isolated cytopenias. The immunophenotype of BCP was determined using 8-color flow cytometry. The expression of BCP markers was evaluated based on normalized median fluorescence intensity calculated for each marker as a difference of positive population and negative reference population (T-cells). For data analysis, the Infinicyt software (Cytognos SL, Spain) was used.

Results: In regenerating bone marrow, significantly higher expression levels of TdT, CD27, CD38, CD10 and CD22 on pro-B-cells, CD27 and CD38 on pre-B-I cells, CD27 and CD34 on pre-B-II cells, CD34 and CD10 on pre-B-III immature B-cells were observed as compared to the respective BCP types of normal bone marrow ($p<0.05$). Furthermore, the expression levels of CD45 on pro-B cells, CD45, cIgM and CD20 on pre-B-II cells and cIgM on immature B-cells were significantly lower in regenerating bone marrow than on the respective BCP types of normal bone marrow ($p<0.05$).

Conclusions: Multiparameter flow cytometry is an important tool for determination of antigenic cells profiles. The developed methodology can be used to determine even slight differences in marker expression. B-cell precursors in bone marrow regenerating after chemotherapy differ significantly in the levels of expression of several antigens when compared to normal B-cell precursors.

PLE-09-02

Update on UK NEQAS MRD programmes

Virgo f Paul
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Whilst flow cytommetric MRD assessment has been widely used in a number of different haematological malignancies in both clinical trials and in individual instituations there has been realtively poor standradisations of techniques used and no form external quality assurance (EQA) of these tests.
UKNEQAS Leucocyte Immunophenotyping have recently established EQA schemes fro a number of diseases.
This talk provides:
An overview of flow MRD utilisation UK, Europe and Worldwide
An overview of considerations in providing EQA scheme s for these assays
An overview of schemes available through UKNEQAS Leucocyte Immunophenotyping
An overview of current scheme/performance issues

PLENARY SESSION 10: UK NEQAS LI FOCUS SESSION

PLE-10-01

UK NEQAS LI Programme Developments

Matthew Fletcher
UK NEQAS for Leucocyte Immunophenotyping, SHEFFIELD, United Kingdom

Matthew Fletcher (Sheffield, UK) and Alison Whitby (Sheffield, UK)

UK NEQAS for Leucocyte Immunophenotyping is an international External Quality Assessment (EQA)/Proficiency Testing (PT) provider hosted by, and is legally accountable to, Sheffield Teaching Hospitals NHS Foundation Trust. We currently provide EQA/PT services for molecular haemato-oncology and flow cytometry for haematological/immunological assays.

As part of our ongoing commitment to EQA and to accommodate the requests of participants, 2016 has seen several new developments in our flow cytometry programmes. Two new Pilot Minimal Residual Disease Programmes for B-CLL and AML have been introduced and the Paroxysmal Nocturnal Haemoglobinuria Programmes have been redesigned to make them more user friendly and to incorporate Monocyte PNH clone determination. Additionally, a new Leukaemia Programme (Leukaemia 1.5) is being developed. The purpose of this session is to give an overview of these programmes and possible future developments.

PLE-10-02

Liam's Laboratory Nightmares

Liam Whitby
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Introduction: UK NEQAS for Leucocyte Immunophenotyping (UK NEQAS LI) is the largest worldwide supplier of flow cytometric external quality assessment (EQA) programmes. Over the last 20 years, they have identified numerous factors that can affect laboratory performance of clinical flow cytometric tests.

Results and discussion: Whilst many causes of aberrant results are relatively simple to identify and correct, there are occasions where the cause is not as straightforward and much more thorough investigations are required in order to assist laboratories in optimising their practices. This session will involve an outline of how to perform a root cause analysis, a guide to the 5 whys and details of some of the more unusual root causes of unsatisfactory performance that have been seen by UK NEQAS LI, and is intended to be of value to any scientist working in a clinical flow cytometric laboratory.

PARALLEL SESSION 1: ADVANCES IN CYTOMETRY 1

PAR-01-01

Exosomes Present in The Human Ovarian Tumor Microenvironments Rapidly Arrest T Cells

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Membrane bound extracellular vesicles isolated from ascites fluid from ovarian cancer patients have biophysical and compositional characteristics similar to vesicles called exosomes. The tumor-associated exosomes inhibit an early activation endpoint (translocation of NFAT from the cytosol into the nucleus) of a significant portion of virus (EBV and CMV) specific CD8+ T-cells that are stimulated with viral peptides presented in the context of Class I MHC. Early and late activation endpoints of peripheral blood CD4+ and CD8+ T-cells (of unknown specificity) stimulated with immobilized antibodies to CD3 and CD28 are also significantly inhibited by the exosomes. The inhibition of the T-cells is induced directly and rapidly (after just a 2h pulse with the exosomes), and occurs coincidentally with the exosomes binding to and internalization by the T-cells. The early arrest in the activation occurs without a loss of viability in the T-cells. The immune suppressive exosomes in the tumor microenvironment and the ability to block their T-cell inhibitory activity represent a potential therapeutic target to enhance the anti-tumor immunity of quiescent tumor-associated T cells, and to prevent the functional arrest of endogenous or adoptively transferred T-cells upon their entry into the tumor.

PAR-01-02

Modeling and Computational Cytometry

Karen Sachs, Garry Nolan
Stanford, PALO ALTO, U.S.A.

Introduction: Signaling networks are the crucial control circuits that regulate homesotasis and determine the fate of cells, in health and disease states. Single cell ctometry provides access to many thousands of observations of the underlying heterogenity in biological systems, and enables statistical analysis due to a large resulting sample size.
Methods: We employ mass cytometry to analyze samples in normal and disease states, and use computational methods, such as probabilistic graphical models, to elucidate pathways and understand network alterations that occur in disease states.
Results: Computational methods employing single cell data will be described, with example applications from drug profiling and disease networks in leukemia. Remaining challenges in computational analysis will also be discussed.
Conclusions: Machine learning contribues tremendously to the ability to extract data from rich single cell datasets. Many computational challenges remain.

PAR-01-03

Clinical cytometry in the cloud: an ESCCA project to bring cytometry data to modern multidisciplinary translational research platforms

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Cytometry constitutes a significant scientific domain of the big data era where the produced data-sets can easily grow beyond the available local management and analysis resources. The continuing increase in volume and complexity is the price to pay for the sustained evolution towards more precise, accurate and detailed results.
Translational research, the critical bridge between lab and clinic, is a fertile big data field where the vast amount of information is being generated in a multidimensional space and cytometry plays a vital role not only as a single dimension, but also as an important member of most others. The complicate and abstract nature of translational research, which can be described as applying ideas, insights, and discoveries to the treatment and prevention of human disease, further intensifies the big data phenomena. Such increased complexity, volume and transdisciplinarity transforms data into a chaotic system that can only be elevated to information by the use of tailor-made computational tools. Discovering new pathways to handle and analyze big data is a one-way street to ensure the discoveries which will advance into human trials have the highest possible safety and efficacy.
The digital evolution of the cloud computational services, aiming to improve accuracy, efficiency, productivity, resources consumption and ease of use, is the emerging platform of modern translational research. Cloud services represent a new approach in computer science where software and hardware become irrelevant and the only concern is the quality of service. Translational research cloud systems [such as transMART] readily process the majority of biomedical data such as clinical, gene expression, small and double region genomic variants, SNP, RNAseq, aCGH, proteomics, RBM, metabolomics and miRNA and enjoy support which extends beyond just reading data values to the interpretation of data properties and understanding the underlying relationships.
Even though it is a vital field of drug discovery, part of most OMICs data production and one of the most requested features by tranSMART community, cytometry is not currently handled by the platform. ESCCA is taking actions to bring cytometry back on top of the research chain, by designing and implementing an international project, open to ESCCA members, that will create the necessary scientific guidelines and tools needed to include cytometric markers in the cloud processes of data management, integration and analysis.

PAR-01-04

A comprehensive phenotypic characterization of bone marrow- and cord blood-derived mesenchymal stromal cells (MSC) unfolds new markers for MSC definition

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Introduction: Mesenchymal stromal cells (MSC) are a heterogeneous population of multipotent progenitor cells used in the clinic for their regenerative and immunomodulatory properties. Although bone marrow (BM) remains the most common source for obtaining off-the-shelf MSC, cord blood (CB) represents an alternative source which can be collected non-invasively and without major ethical concerns. To date, comprehensive and comparative studies characterizing MSC phenotype are still lacking. We aimed at providing for the first time to our knowledge an in-depth immunophenotypic analysis of BM- and CB-derived MSC.
Methods: BM-MSC (n=4) were isolated and expanded as bulks (n=4) or single clones isolated from the bulk culture (n=3). CB-MSC (n=6) were isolated and expanded as single clones in 5/6 samples. Cell phenotype was investigated by using the BD Lyoplate Human Screening Panel (BD-Bioscience), a system covering 242 antibodies and related isotype controls. Chondroitin, vimentin, alpha-SMA and CD276 antibodies were added to the original panel. Cell staining was performed according to producer's recommendations. Samples were acquired using BD FACSCanto II (BD-Bioscience). Data were analyzed by FlowJo software (TreeStar). Fold increase with respect to isotype control (FI), percentage of positive cells (%pos) and robust Coefficient of Variation (rCV) were used to describe each marker. The FI Log2 transformation was performed (Log2FI).
Results: Starting from 246 markers we selected those (n=59) showing the smallest variation between samples (CV of Log2FI≤0.5). Then, based on unsupervised hierarchical cluster analysis (HCL) we have identified 4 groups of markers according to their relative expression intensity (high, intermediate-high, intermediate-low, low). To find the best MSC markers for both cell sources, we then focused on the group of the high expressed markers (alpha-SMA, Beta-2-microglobulin, CD105, CD13, CD140b, CD147, CD151, CD276, CD29, CD44, CD47, CD59, CD73, CD81, CD90, CD98, HLA-ABC, Vimentin). Marker selection was refined using %pos and rCV in order to identify those markers with the highest %pos and the narrowest distribution (rCV) for each sample. Accordingly, all high expressed markers excepting CD140b and alpha-SMA were suitable for the specific identification of *in-vitro* expanded MSC from both sources.
Conclusion: We have identified specific markers shared and strongly expressed by BM- and CB-derived MSC that could complement the minimal panel proposed for *in-vitro* MSC definition. Once validated on a greater number of cases, this analysis will allow the identification of markers differentially expressed by the two MSC sources.

PAR-02-01

Application of flow cytometry for the evaluation of bone marrow dysplasia in myelodysplastic syndromes in the WHO era

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Myelodysplastic syndromes (MDS) are a heterogeneous group of disorders clinically characterized by peripheral cytopenia an increasing risk of evolution into acute leukemia. The biologic hallmark of MDS is marrow dysplasia which indicates a defective capacity for self-renewal and differentiation of hematopoietic progenitors and relies on various morphological abnormalities. Immunophenotyping has been recently proposed as a tool to improve the evaluation of marrow dysplasia. An first contribution of flow cytometry in the setting of MDS may derive from the study of blasts cells and CD34 compartment, that has diagnostic and prognostic relevance. Moreover, evidence of granulocytic dysplasia as defined by WHO criteria is present in about 50% of MDS. Most significant alteration include hypo-/agranularity on myeloid cells, presence of pseudo-pelger neutrophils and increased prevalence in bone marrow of myeloid cells in the earliest stage of maturation, that significantly affected detection of physical parameters by flow cytometer. In addition, defective capacity for self-renewal and differentiation by myelodysplastic stem cells relies on various abnormalities of antigen expression on granulocytic cells, which may be easily detected by flow cytometry. Erythroid dysplasia is found in almost all patients with MDS and is the only morphological abnormality in those with refractory anemia. An important problem in this group of diseases is the possibility of misdiagnosis of MDS due to overinterpretation of dyspoiesis that is secondary to a nonclonal disorder. A dysregulation of CD71 expression is the most frequent abnormalityes reported in MDS by FC studies. Interestingly, CD71 is receptor for transferrin. It is well known that iron metabolism is clearly important in erythroid cells for heme production and is peculiarly perturbed in MDS. So, the evaluation of proteins of iron metabolism could represent a promising target to overcome the limited availability of antibodies specific for erythroid lineage. In addition FC analysis might be helpful in characterization of the sideroblastic anemias. Sideroblastic anemias are a group of disorders characterized by ring sideroblasts, that is, red cell precursors with mitochondrial iron accumulation. Iron deposited in perinuclear mitochondria of ring sideroblasts is present in the form of a particular type of ferritin, called MtF and that this latter might be a specific marker of sideroblastic anemia. By using a specific marker against MtF, FC analysis confirmed that MtF expression is closely related to the presence of ring sideroblasts in bone marrow.

PAR-02-02

Experience in national harmonisation for PNH detection

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Introduction: Paroxysmal nocturnal haemoglobinuria (PNH) is a rare disorder with high mortality without appropriate diagnosis and treatment. The condition manifests as a consequence of the glyco-phosphatidyl-inositol (GPI) anchor deficiency.
Several publications and guidelines have been produced advising on best practice approaches for analysis of GPI deficient cells by flow cytometry. We formed a multicentre group in 2011; our aim was to review the published guidance and demonstrate that a harmonised protocol is reproducible in laboratories with different equipment and expertise.
Methods: Detailed red blood cell (RBC) and white blood cell (WBC) PNH testing protocols were determined through consensus discussion following review of published protocols and local laboratory methods. Methods were validated in multiple rounds of inter-laboratory testing (4 x RBC and 4 x WBC).
More than 100 normal samples were tested locally to determine assay background levels. 20 abnormal samples (range 0.01 -99.9%) and 9 normal samples were tested in a total of 8 inter-laboratory sample send outs. All data was extensively reviewed by the group after each round of testing. A number of method improvements were identified, implemented and re-validated in subsequent testing rounds.
Results: Levels of GPI-deficient populations in normal samples were <0.01% in all samples tested and all laboratories correctly identified samples with PNH clones with high precision. We were also able to evidence assay performance characteristics including clone and conjugate performance, precision, linearity, sensitivity, instrument setup, data analysis and analyte analyte stability.

Conclusion: We have been able to establish a consensus protocol based upon published data and provide evidence this is reproducible in a multi-centre setting. We are now seeking to extend the group to include more laboratories.

PAR-02-03

CLINICAL IMPACT OF ONCOGENETIC PROFILES IN SYSTEMIC MASTOCYTOSIS WITH AN ASSOCIATED HEMATOLOGICAL NON-MAST CELL DISEASE.

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Introduction: In a substantial fraction of systemic mastocytosis (SM) patients, SM coexists with an associated clonal hematological non-mast cell (MC) lineage disease (AHNMD). Most SM-AHNMD patients carry KIT mutations and AHNMD-associated genetic alterations; however, there is limited information about the frequency and clinical impact of the coexistence of both types of genetic/molecular alterations in distinct bone marrow (BM) cell compartments. We report on the clinic-biological, genetic and prognostic features of 65 SM-AHNMD patients classified into three different ontogenetic groups based on the pattern of involvement of BM MC, AHNMD tumor cells and other residual BM cells, by both the KIT mutation and AHNMD associated cytogenetic/molecular alterations.
Methods: We studied 65 SM-AHNMD patients grouped into SM-AHNMD cases with: i) unrelated genetic alterations; ii) shared KIT mutation in BM-MC and AHNMD tumor cells, in the absence of AHNMD-associated genetic alterations in BM MC, and; iii) shared AHNMD-associated genetic alterations.*Cell Purification.* Purification of specific BM cell populations was performed using a FACSAria flow cytometer (BD).*Interphase fluorescence in situ hybridization (iFISH).* iFISH studies aimed at detection of t(9;22), t(8;21), inv(16), 11q abnormalities, -5/del(5q), -7/del(7q), del(20q), trisomy 8, nulisomy Y, trisomy 12, del(17p13.1), del(13q14), t(14q32), t(18q21), t(11;14), t(3q27) and del(6q21) were performed on interphase nuclei from different FACS-purified and methanol/acetic fixed 3/1 (v/v) cell populations.*KIT mutational analysis*. The *KIT* D816V mutation was assessed in genomic DNA from FACS-purified cell populations, using a polymerase chain reaction and peptide nucleic acid-clampingtechnique.
Results: Overall, patients with shared AHNMD-associated genetic alterations showed a significantly poorer progressionfree survival (PFS) and overall survival (OS) vs. the other two groups ($p< 0.01$).The pattern of involvement of BM cell compartments other than MC by the KIT mutation and the subtypes of SM and AHNMD, were also relevant prognostic factors in the univariate analysis ($p< 0.01$). Multivariate analysis confirmed that the best combination of independent prognostic factors for OS and PFS were the pattern of involvement of BM cells by the KIT mutation ($p<0.001$ and $p< 0.01$, respectively) and the oncogenetic subgroup of AHNMD ($p=0.02$ and $p< 0.01$, respectively) together or not with the type of AHNMD (HR, 27.9; $p< 0.001$), respectively.
Conclusion: Disease behavior and patient outcome depend on the underlying oncogenetic profile of tumor MC and AHNMD cells, in addition to the specific diagnostic subtypes of SM and AHNMD.
Coexistence of the KIT mutation and AHNMD-associated genetic markers in BM MC and AHNMD cells is an adverse prognostic factor in SM-ANMD.

PAR-02-04

Multicenter validation of a simplified 3-color FCM assay for PNH screening

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Introduction: According to the international guidelines, state-of-the art PNH testing requires complex and costly single-tube 5- or 6-color or multiple tube 4-color assays. However, PNH clones may be detected in only 1/30 to 1/100 of the tested samples, depending on the robustness of the clinical suspect. As a consequence, screening more or less selected cases for PNH clones with complex multicolor assays may be very expensive, with the risk to incur into cost-restriction policies and to fail to detect the presence of the disease in many patients. In this multicenter study we have validated a cheap and simplified one-tube 3-color FCM assay to be used as an extensively applicable and sustainable screening test for PNH.

Methods: Six expert laboratories, equipped with FACSCanto II routinely calibrated with the BD OneFlow beads, were sent 10 fresh blind samples containing spiked PNH clones from 0% to 35%, to be analyzed in parallel with a centrally provided 6-color pre-titrated cocktail (FLAER Alexa488/ CD24PE/ CD45 PerCP-Cy5.5/ CD64PECy7/ CD15APC/ CD14APC-Cy7) and a simplified 3-color mixture (FLAER Alexa488/ CD45 PerCP-Cy5.5/ CD15APC), along with a strict analysis protocol requiring the collection of 250,000 CD45+ events and a common FACSDiva template. Moreover, one lab also performed assay precision and sensitivity tests with the simplified panel on replicate spiked samples, from undiluted down to 1:10,000.

Results: The performance and agreement comparison (Regression and Bland-Altman analyses) between 6-color and 3-color assays gave: Granulocyte PNH clone (range 0-22%) Y=1.01X-0.002, rsq=0.998; Mean Diff +0.008%, Limits of Agreement +0.05/-0.05%. Monocyte PNH clone (range 0-35%) Y=0.97X+0.25, rsq=0.955; Mean Diff -0.58%, Limits of Agreement +2.02/-3.18%. Sensitivity and precision of 3-color assay was 0.01%, CV<3% for Granulocytes and 0.1%, CV<5% for Monocytes, respectively.

Conclusion. A simplified 3-color PNH test has been validated in a multicenter study accomplished with a high degree of instrument standardization. Precision and sensitivity of the simplified test were comparable to the more complex and expensive state-of-the-art 6-color assay. The cheaper simplified assay can be extensively applicable for screening purposes. A centralized reanalysis of the multicenter dataset to evaluate the performance of a further simplified 2-color assay (FLAER/CD15 or FLAER/CD45) is still under way.

PARALLEL SESSION 3: IMMUNOLOGY 1

PAR-03-01

B-cells in immunodeficencies

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Introduction: The function of B cells is to produce antibodies that protect the organism from infection. Patients with immunodeficiency are unable to produce antibodies and have increased susceptibility to infection. Administration of immunoglobulin substitution therapy only partially reduces the severity of the symptoms. Physiologically protective antibodies are produced by memory B cells and plasma cells generated in response to vaccination or infection. There are two main types of memory B cells, IgM and switched memory B cells.
Patients with immunodeficiency lack memory B cells. The symptoms of immune impairment are different depending on whether only one of the two subsets is reduced or absent. This suggests that switched and IgM memory B cells execute different and non-interchangeable functions.
Results and discussion: We studied memory B cells in children of different ages, in peripheral blood and spleen and compared them to children born asplenic or unable to build germinal centers. We show that whereas switched memory B cells are exclusively generated in the germinal centers at all ages, in spleen and peripheral blood, three distinct types of IgM memory B cells exist. Innate memory B cells, the largest pool in infants, generated in the spleen by a germinal center-independent mechanism. With age, if the spleen is present and germinal centers are functional, innate memory cells become remodelled increasing SHM. The third type is produced in the germinal center as a by-product of high-affinity switched memory B cells. The B-cell memory developmental program is implemented during the first 5-6 years of life establishing a competent adult repertoire by the age of 7.

PAR-03-02

Long-lived Plasma Cells and Vaccination

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Plasma cells (PCs) are heterogeneous in terms of function and lifespan. Long-lived PCs play central roles in immunity and autoimmunity, and may represent the cell of origin for myeloma. Neoplastic PCs were originally characterized by a lack of CD19 expression, however it is now known that polyclonal CD19Neg PCs constitute approximately 15-35% of total PCs in the general adult population. Whether human CD19Neg PCs are “aged” cells that have gradually lost CD19 expression, or arise during the initial process of PC differentiation is not known.
Human bone marrow CD19Neg PCs are phenotypically distinct from normal CD19Pos PCs with weaker CD95 and stronger BCL2 expression. Normal CD19Neg PCs share some characteristics with neoplastic PCs including variable expression of CD28, CD56, and CD45, but neoplastic PCs are reliably distinguished from normal CD19Neg PCs by differential expression of CD27, CD81 and CD117 as well as CD56 in some cases. After effective myeloablative therapy CD19Pos PCs recover in all patients, while CD19Neg PCs can remain depleted for several years. Therapeutic B-cell depletion with alemtuzumab results in loss of CD19Pos PCs within 3 months whereas CD19Neg PCs persist.
High-throughput sequencing demonstrates little overlap between the IGHV repertoire with only 2.1 to 3.7% of unique IGHV@ rearrangements shared between the CD19Pos and CD19Neg fractions, suggestive of a largely discrete immunoglobulin repertoire in the two fractions at steady state.
CD19Neg plasmablasts are detectable in peripheral blood at steady state and during the acute response to influenza vaccination in healthy donors. These are antibody-secreting cells (ASCs) and during the acute response to influenza vaccination CD19-positive, CD19-low and CD19-negative ASCs secrete vaccine specific antibody as demonstrated by ELISpot.
Using in vitro models which mimic both T-dependent and T-independent differentiation, we find that the CD19-negative state is established at the plasmablast to PC transition, that CD19Neg PCs increase as a percentage of surviving PCs over time, and that CD19Neg and CD19Pos PCs can be maintained independently.
In conclusion, CD19Pos and CD19Neg PCs are biologically distinct with different immunoglobulin repertoires and production kinetics consistent with CD19Neg PCs having greater potential lifespan. CD19Neg PCs are established both in vivo and in vitro during the initial process of PC differentiation, supporting a “decision”-based model for CD19Neg PC generation. Identifying the developmental pathway of normal CD19Neg PC is likely to improve our understanding of vaccination response as well as the pathobiology of both plasma cell neoplasms and autoimmune conditions.

PAR-03-03

Distribution of peripheral blood (PB) B cell subsets through life

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Introduction: Humoral immunocompetence has been traditionally evaluated via soluble immunoglobulin (Ig) levels; however, Ig levels only partially reflect B-cell competence. We have developed an approach for quantification of subsets of maturation and isotype associated B-cells by flow cytometry (FCM). Here we describe the distribution of such B-cell populations through life.

Methods: PB B-cells from 192 healthy donors (age: 2 days to 89 years) and 16 cord blood (CB) samples classified into immature, naïve and memory B-cells (MB), plus plasma cells (PC) were analyzed. Based on their Ig isotype, MB and PC were subclassified into IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM and IgD cells using 12-color FCM. Soluble Ig plasma levels were analyzed in parallel by nephelometry.

Results: In CB and newborn PB, most B-cells corresponded to immature (138±73 and 191±155 cells/µL, respectively) and naïve (454±220 and 349±230 cells/µL, respectively) B-lymphocytes. In both types of samples, MB-cells expressing most isotypes (IgG1, IgG2, IgG3, IgA1 and IgA2) were also detected but at extremely low counts (<</u>0.5cells/µL). In contrast, PC were absent in CB whereas detectable in newborns (mostly IgM+ PC).

Overall, IgG1, IgG4, IgA1, and IgA2 PC raised first at 6-12 months (11±5, 0.2±0.4, 24±14, and 5±3 cells/µL, respectively), subsequently followed by IgM, IgG2 and IgG3 PC at 1-2 years (y) (11±12, 1.2±1.3 and 0.9±1 cells/µL, respectively) and IgD PC (0.7±0.7cells/µL) at 2-5y. Thereafter, all isotypic subsets of PC decreased in PB during childhood, reaching stable levels at adulthood.

PB MB peaked later than PC. Thus, the highest IgMD, IgG1, IgG3, IgA1, and IgD MB-cell counts were detected at 2-5y (97±46, 52±31, 8±5, 15±7 and 3±2 cells/µL, respectively), decreasing thereafter. In contrast, maximum IgG2, IgA2 and IgG4 MB-cells were only reached at adulthood (30-40y, 50-60y and 50-60y: 8±5, 6±4 and 1±1 cells/µL, respectively), their levels remaining relatively stable thereafter.

For all different isotypes, maximum Ig plasma levels were reached later than their corresponding cell compartments.

Conclusions: Multiparameter FCM can dissect the PB B-cell compartment into >30 different maturation-associated and Ig-isotype specific B-cell and PC subsets. Using this approach we observed that during B-cell reconstitution after birth, PC and MB numbers increase in PB faster than their corresponding plasma Ig levels, for all different Ig isotypes. This provides a frame of reference for the analysis of humoral B-cell responses in patients with primary antibody-immunodeficiencies, as well as other immune-related disorders.

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PAR-03-04

Cathepsin S inhibitor target engagement and dose selection for autoimmune diseases

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Introduction: Major histocompatibility complex (MHC) class II-restricted autoantigen priming of CD4+ T cells is thought to play a role in the pathogenesis of a number of autoimmune diseases. This has led professional antigen presenting cells (APC) interaction with CD4+ T cells to become a target for various therapeutic approaches. MHC class II molecules are initially associated to the invariant chain (Ii), a chaperone molecule that needs to be degraded before (auto)antigenic peptides can be acquired. Inside B cells and dendritic cells, cathepsin S (CatS) is the single enzyme responsible for the last step of the Ii proteolytic cascade, mediating the cleavage of Iip10 to form CLIP. As a result, therapeutic targeting of CatS has focused on Iip10 as an indication of target engagement and quantitative readout of drug exposure. Here we report the development of a pharmacodynamic (PD) biomarker assay using flow cytometry to detect intracellular Iip10 accumulation in professional APC and its application to a Phase 1 clinical study.
Methods: PBMC enriched from the blood of healthy human volunteers or cynomolgus monkeys were either incubated for 20h in the presence of increasing concentration of CatS inhibitors in an *ex vivo* assay, or stained immediately if subjects had been administered an oral dose of CatS inhibitor. Intracellular Iip10 accumulation in combination with immune lineage markers was measured by multiparametric flow cytometry.
Results: *Ex vivo* incubation of CatS inhibitors with PBMC yielded a dose-dependent accumulation of Iip10 in B cells. Stain index values derived from mean fluorescence intensity (MFI) measurements allowed for direct comparison between time-points and subjects, revealing a high degree of heterogeneity in maximum response among healthy volunteers. Simultaneously, IC50 derived from dose response curves could be used to classify the potency of inhibitory compounds. An additional proof-of-concept study in animals treated with a CatS inhibitor demonstrated that the assay could measure PD activity *in vivo*.
Following a successful development phase, the assay was applied to a human clinical study of CatS inhibition in healthy volunteers. Dose-dependent accumulation of Iip10 could be measured specifically in B cells, providing clear information on target engagement by the inhibitor administered orally. The assay also helped characterize the dose-response relationship.
Conclusions: This robust assay can provide confirmation of *in vivo* target engagement for CatS inhibitors. IC50 and maximum response values derived from *ex vivo* data combined with PD read-out from clinical data allow optimal dose selection for maximal biological effects in humans.

PARALLEL SESSION 4: HEMATOLOGY-ONCOLOGY 2

PAR-04-01

The value of novel B-cell markers in B-Lymphoproliferative Disorders' diagnosis

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Introduction: Since the publication of the revised Matutes CLL scoring system (1997), advances in instrumentation and fluorochrome availability have facilitated a multicolour approach to diagnosis. Whole genome sequencing has identified new antigens of interest leading to the development of new antibodies for diagnosis of B-cell lymphoproliferative disorders (B-LPDs).
This presentation aims to review the role of cell markers and new software in the differential diagnosis of B-LPDs especially in the context of the revised CLL scoring system and by reviewing the literature, to reexamine which prognostic markers remain of value in CLL. This was performed in the context of testing the value of our current laboratory practice.
Methods: We retrospectively reviewed 114 B-LPDs cases referred to the Royal Marsden Hospital at diagnosis. Eight colour antibody panels: a modified Euroflow consortium's LST tube and a B-cell tube were used. Diagnosis was made based on morphology and the revised Matutes score. FISH results were included in atypical cases. Our results were comparable with published data. Of specific interest were the newer antibodies: CD200, CD43, and RORI. The utility of CD38, and ZAP-70 were also examined.
Results: CD200, CD43 and RORI monoclonal antibodies were of value in differentiating CLL from MCL. In HCL, CD200, CD103 CD123 were important in distinguishing this disorder from other villous disorders. B-NHLs and LPL proved more difficult to differentiate.
Literature review revealed that CD38 and CD49d expression by flow cytometry remain useful prognostic indicators in CLL but the expression of ZAP-70 does not appear to provide a robust surrogate for IGHV immunoglobulin mutational status as previously suggested.
Conclusion: We conclude that the value added by novel monoclonal antibodies is an important contribution to diagnosis of B-LPDs but has its limitations. This is true for B-NHLs where the availability of markers to accurately classify LPL, DLBCL , SMZL and villous disorders, by flow cytometry is currently inadequate. Histology remains essential to differentiate these.
Multiparametric flow cytometry is of use to exclude lymphocytosis and to classify a small subset of B-LPD cases and it's strength remains accurate low level detection.
The role of ZAP70 as a surrogate marker for mutational status in CLL has been surpassed by molecular studies, this coupled with the technical challenges related to its detection has resulted in this test being discontinued in our laboratory.

PAR-04-02

MRD testing for B-lymphoblastic leukemia after anti-CD19 therapy

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Introduction: Minimal residual disease (MRD) is an important prognostic factor in B-lymphoblastic leukemia (B-LL). B cells at almost all maturational stages express CD19. For this reason, CD19 is often employed as a starting point for B cell identification in gating strategies for B-LL MRD detection. The use of anti CD19 therapy for B cell neoplasms is increasing. Use of such therapies may lead to a decrease in CD19 expressing B cells. Additionally, patients exposed to such therapies may relapse with CD19 negative disease. In the setting of anti CD19 therapy, alternative strategies are needed for B cell detection. This presentation will discuss a novel combination using expression of CD22 or CD24 for B cell identification as a starting point for B-LL MRD detection.
Methods: The performance characteristics of this combination will be described in both normal and abnormal samples. Lessons learned during the validation process that may have implications for MRD detection will be explored.
Results: This combination showed good correlation with the standard flow cytometric method for MRD detection and was able to identify CD19 positive and CD19 negative residual B-LL. Cases will be presented to provide examples of the performance of this combination and to illustrate potential pitfalls.

Discussion: The use of targeted therapy is growing. When designing flow cytometric strategies for population identification and characterization, it is critical to be aware of what targeted therapies may be used, and to consider how such therapies may impact the immunophenotype of the neoplastic population and the background normal cells.

PAR-04-03

High frequency of central nervous system involvement in blastic plasmacytoid dendritic cell neoplasm at diagnosis and role of intrathecal therapy

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Introduction: Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a rare aggressive myeloid neoplasm which shows a high rate of central nervous system (CNS) recurrence (≥30%) and a short overall survival (OS), usually <1 year. Despite this, screening for CNS involvement is not routinely performed at diagnosis and intrathecal (IT) prophylaxis is not regularly administered in these patients. In this study, we analysed the incidence of CNS involvement in BPDCN patients at diagnosis and evaluated the impact of intrathecal prophylaxis administration on patient outcome.
Methods: 41 stabilized cerebrospinal fluid (CSF) samples from 13 consecutive BPDCN patients were evaluated for the presence of CNS involvement by flow cytometry immunophenotypic studies. Cases were evaluated at diagnosis (n=10) or at relapse/progression (n=3) and subsequently, after IT therapy.
Results: Despite none of the patients presented with neurological symptoms at disease staging, CNS involvement was detected in 6/10 cases evaluated at diagnosis and 3/3 studied at relapse/progression. Detection of tumour cells in the CSF was associated with ≥20% bone marrow involvement by neoplastic cells. BPDCN patients evaluated at diagnosis received IT treatment -either CNS prophylaxis (n=4) or active therapy (n=6)- and all but one remain alive (median follow-up of 20 months). In contrast, all 3 patients assessed at relapse/progression died. Follow-up CSF samples obtained after IT therapy showed absence of tumour cells in 6/6 CSF^+ cases studied at diagnosis, either after one -5/6 cases- or 4 doses of therapy (6/6 cases). The potential benefit of IT treatment administered early at diagnosis was further confirmed in a retrospective cohort of another 23 BPDCN patients. Multivariate analysis of prognostic factors performed in the whole patient cohort (n=36) showed that IT prophylaxis/treatment at diagnosis was the only independent (favourable) prognostic factor for CNS-recurrence-free survival and OS.
Conclusions: Our findings show that BPDCN patients studied at diagnosis frequently display CNS involvement; moreover, these data also indicate that treatment of occult CNS disease might lead to an improved outcome for BPDCN. These results suggest that the CNS could be a persistent blast-cell sanctuary in BPDCN patients with leukemic presentation, due to the limited power of cytostatic drugs to cross the blood-brain barrier into the CSF and brain parenchyma. This reservoir of leukemic cells may also contribute to the high rate of bone marrow/systemic disease recurrence observed in these patients.

PAR-04-04

Seventeen parameter, 15-marker, 10-color single tube flow-cytometric assay for screening & diagnosis of chronic lymphoproliferative neoplasms

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Introduction: Detection of phenotypically aberrant and clonal lymphocytes is the hallmark of flow-cytometric (FC) detection of chronic lymphoproliferative disorders (CLPD). However, specimens like CSF, other body-fluids are usually paucicellular and do not allow use of multiple-tube antibody-panel. Similarly, in bone-marrow samples with minimal involvement it is difficult to trace the tumor cells using multiple-tube antibody-panel. Moreover, multiple tubes increase the cost-per-test which is a practical concern for the laboratory. Six and 8-color lymphoma screening tubes are used widely. We standardized a 15-marker 10-color single-tube lymphoma screening FC-assay (LST) that allows detection of phenotypically aberrant & clonal population of mature B-cells, T-cells and plasma cells.

Methods: We studied 174 samples (103-bone marrows, 4-peripheral blood, 17-CSF, 11-ascitic fluids, 34-pleural fluids, 2-vitreous fluids and 3-lymph node aspirates) using 15-marker-LST on Navios-flow-cytometer and data was analyzed using Kaluza-software. LST includes antibody-fluorochrome (clone) as follows: CD3/CD14-BV510 (OKT3/M5E2), CD19/TGRD-BV421 (HIB19/B1), CD8/Kappa-FITC (B9.11/Polyclonal), CD7/Lambda-PE (8H8.1/Polyclonal), CD34-ECD (581), CD4/CD20-PERCY-CY5.5 (13B8.2/B9E9), CD5-PC7 (BL1a), CD10-APC (ALB1), CD45-APCAF700 (J.33) and CD38-APCAF750 (LS198-4-3). To evaluate the efficacy of LST, we compared the results of LST with routine 8-color 3-tubes CLPD-assay in 40 samples (20-involved & 20-uninvolved). Study was conducted in Tata Memorial Centre, Mumbai, India.

Results: Of 174 cases, 38 cases were positive for B-cell neoplasm (7-CLL, 4-low-grade B-cell NHL, 9-DLBCL, 3-BCPALL, 1-HCL, 6-FCL, 1-PMBCL and 2-plasma cell neoplasm) and 2-T-NHL (PTCL-NOS). Minimum levels of abnormal-cells detected in LST were 0.36% (median 10.6%; range 0.4-90.9%). On Pearson-correlation of percentages of lymphocyte subsets (B, T & NK-cells) in 20-uninvolved samples between LST & routine 8-color CLPD-assay, an excellent correlation was found (r = 0.91 & p-value <0.01). Similarly, there was high correlation of percentage of abnormal or clonal B/T/plasma cells in 20-involved samples (r = 0.98 & p-value <0.001). Cost/assay of LST was 31-USD far less than 60-USD of routine 3-tube 8-color CLPD-panel.

Conclusion: We established a 17-parameter, 15-marker, 10-color single-tube lymphoma screening assay that can successfully establish B-cell clonality and detect aberrant B/T/plasma cells. It can be effectively used in paucicellular-samples like CSF and samples with minimal involvement. Hence, it is very useful in the diagnosis & staging of CLPD. LST effectively reduces the cost-per-test and turn-around time. Its pivotal role in paucicellular samples makes LST the assay-of-interest.

PARALLEL SESSION 5: ADVANCES IN CYTOMETRY 2

PAR-05-01

Advances in cytometry from a core manager perspective

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Cytometry is a field in continuous growth and expansion. New instruments are coming to the market with incredible improvements in terms of sensitivity, capabilities, portability, stability, etc. It is quite interesting to look back just a few years ago and find out that running a 3 colours panel was considered as performing multicolor flow cytometry, and nowadays there are instruments to measure +50 markers at the same time. The constant demand of certain sectors (mainly clinical and biopharma) to the manufacturers for faster and more precise instruments is pushing them to explore and invest in new technologies. The evolution in the instrumentation walk side by side together with: the appearance of new reagents and dyes (Quantum dots, Brilliant Violets, Live/Dead Fixable dyes,…); the development on the data analysis and interpretation; the improvements in the standardization; considerations for a multicolor panel designs,… With every step on this fascinating evolution of cytometry new fields are showing up waiting to be explored, stopped in multiple occasions due to new technical limitations.
There is no doubt that from a technical point of view, we are living an amazing technological revolution in the field of cytometry. But for many labs and research groups is not easy to be updated of every single technology. Core facilities are without any doubt a very economic option for the institutions to warranty access to their members to the latest instrumentation. These services and facilities exist to enhance and expand collaborative capabilities, provide state-of-the-art resources for faculty research, training for students and attract collaborations with external partners. Share resources labs are managed by experts which will provide technical expertise, consultation and training. Even more the personnel in charge of these facilities are eager to explore how best to use the new tools available in the market to provide their users the correct answer and to contribute in the advancement of cytometry.

PAR-05-02

In vitro evaluation of the potential anticancer activity of new memantin co-drugs on rat C6 glioblastoma cells

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Introduction: Memantin, an anticancer compound which acts by modulating glutammate concentration, has been conjugated to phenyl butirric acid, ferulic acid and acetyl phenylalanine, well known histone deacetylase inhibitors. Histone deacetylases (HDACs) overexpression is associated with higher tumor grade, advanced clinical stage and poor survival. This study was aimed to evaluate the *in vitro* activity of memantin-derived histone deacetylase inhibitors and the molecular mechanisms underlying their biological activity in C6 rat glioma cell line.
Materials and Methods: Cells were treated with 30 µM of memantin conjugated with ferulic acid (co-drug 5) and acetyl phenylalanine (co-drug 7). Cell growth was measured by MTT assay while cytotoxicity was assessed by means of LDH release. Cell cycle analysis, reactive oxygen species (ROS) detection and histone H3 hyperacetylation and apoptosis occurrence were performed by flow cytometry.
Results: MTT analysis discloses a significant reduction upon 24 h of treatment with 30 µM co-drug 5 and co-drug 7 when compared to DMSO/control samples and the respective enantiomers. Moreover, in the presence of co-drug 5 and 7 the LDH assay highlights cytotoxicity occurrence starting from short period of exposure (6 h) which raises up after 15 h of treatment. The flow cytometric analysis of the cell cycle shows C6 cells blocked in the G1 phase after 15 and 24 h of exposure, manly when co-drug 5 is present. After 24 h of treatment an increase in the apoptotic population is observed, significantly with co-drug 5. This biological effect is supported by an observed CCP32 cleaved fragment increased expression at the molecular level. Finally the inhibition in the acetylation of histone H3 was evaluated by means of flow cytometry. The analysis discloses that co-drug 5 seems to be the most efficient in inhibiting H3 deacetylation and the maximum effect is observed after 24 h of treatment. Since the histone 3 deacetylase inhibition triggers oxidative stress, analysis of ROS production was performed, showing an increase upon 6 h of treatment with co-drug 5.
Conclusions: All in all, our study shows that ferulic acid-memantin and acetyl phenylalanine-memantin are effective compounds in modulating the biological and molecular responses in rat C6 glioblastoma cells and they are promising candidates as antitumor compounds.

PAR-05-03

The Proliferation Index of bone marrow cells by flow cytometry assists the diagnostic of Chronic Myelomonocytic Leukemia vs. Monoblastic/Monocytic Leukemias

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Introduction: Overlapping features hamper the differential diagnosis of chronic myelomonocytic leukemia (CMML) and acute monoblastic/monocytic leukemias (AMML). We investigated the proliferation index (PI) of different bone morrow (BM) cell subsets as a potentially tool for discrimination of these diseases.
Methods: In 133 BM samples corresponding to healthy subjects (NBM; n=74), CMML (n=24; CMML-1, n=19 and **C**MML-2, n=5) and AMML (n=36; monoblastic leukemia, n=28 and monocytic leukemia, n=8), the PI (percentage of S+G2/M cells) was studied among the more immature cell component (i.e. CD34+ cells and/or leukemic cells) and residual monocytic, neutrophil and erythroid lineage cells by (5-color) flow cytometry, including the DRAQ5 stain.
Results: In NBM the highest PI was depicted by erythroid lineage cells, followed by $CD34^+$ precursors, neutrophil and monocytic cells (median PI of 28%, 15%, 5% and 4%, respectively).
The analysis of the more immature cell component revealed that CD34+ cells from NBM and CMML had a similar PI (15% vs. 16%; respectively), while it was decreased in AMML (CD34+) leukemic cells, compared to NBM (7% vs. 15%; $p<0.001$), decreasing from monoblastic to monocytic leukemias (8% and 4%, respectively; $p<0.001$). As in NBM, the proliferation of CD34+ cells from CMML-1 (PI of 16%) remained higher than the PI of leukemic cells from both AMML entities ($p<0.001$), while it was similar in CMML-2 and monoblastic and monocytic leukemias (PI of 8% vs. 8% and 4%; $p>0.05$). A trend to a lower PI among leukemic cells is observed in monocytic leukemia, which might help the differential diagnostic vs. CMML-2.
The PI of the erythroid lineage decreased in AMML compared to NBM (23% vs. 28%: $p=0.05$) due to monocytic leukemia, that showed the lower erythroid PI vs. NBM, monoblastic leukemia and CMML-1 (15%, 28% vs. 27% and 30%; $p\leq 0.002$); while it was not significantly lower vs. CMML-2 (15% vs. 24%; $p>0.05$).
Comparing to monocytic cells, BM leukemic cells from AMML depicted an increased proliferation vs. NBM and CMML (7% vs. 4% and 3%; $p\leq0.01$), due to monoblastic leukemia cells (PI of 8%), while the PI of monocytic leukemia cells was similar to maturing monocytes from NBM and CMML (PI of 4%).
Conclusion: Leukemic cells from AMML showed decreased proliferation vs. CMML and NBM. Although it needs confirmation in larger series, the detection of low levels of proliferation among leukemic cells, erythroid and monocytic cells might help the diagnosis of CMML-2 vs. monocytic leukemias.

PAR-05-04

This lecture has been cancelled.

PAR-05-05

Providing an efficient method to perform differential cell counts in BAL fluid using an 8-color flow-cytometry panel

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Introduction: Phenotyping and differential counts of immune cells in bronchoalveolar lavage (BAL) fluid are helpful in diagnosing interstitial lung disease (ILD). Conventionally, differential cell counts are performed by microscopy of cytospin samples. The altered morphology of cells in BAL fluid makes the morphologic count a time and labor intensive method, which requires experience. Furthermore, morphology does not allow differentiation of T-, NK- and B-cells, and mast cell identification requires a separate staining. On the other hand, flow cytometry assess the patterns of expression of lineage specific antigens on the cells, making it possible to differentiate cell subsets regardless of morphological alterations. Currently, flow cytometry is mainly used to establish the CD4/CD8 ratio of T-cells in BAL fluid. However, all major immune cell subsets can be determined using the appropriate antibody panel, thus improving diagnostic accuracy. Published results from our lab showed a better discrimination between hypersensitivity pneumonitis and sarcoidosis using the fraction of activated CD8+ T-cells and NK-T-cells. Our lab have 20 years' experience performing flow cytometry of immune cells from BAL fluid using 4-6 color panels. We propose an 8-color/ 2 tube combination of monoclonal antibodies suitable for identification of the most relevant cell populations in BAL fluid.

Methods: BAL fluid fractions of cell populations were determined by flow cytometry using FACS Canto II flow cytometer, FACS DIVA (BD Biosciences, Mountain view, CA, USA) and Infinicyt (Cytognos, Salamanca, Spain) software. A minimum of 50 000 cells were analyzed. Leucocytes and epithelial cells were distinguished using CD45 and CD326. Subsets of T-, NK-T and NK-cells were identified using CD3, CD4, CD8, CD16, CD56, B-cells using CD19, macrophages/monocytes using CD45, HLA-DR and CD14, eosinophils using Siglec-8, neutrophils using CD16, and mast cells using CD117. Activation of T cell subsets were evaluated by HLA-DR expression.
Results: Staining with the new 8-color panel ensured distinct separation of the 10 cell populations CD4+ T-cells, CD8+ T-cells, NK-T cells, NK-cells, B-cells, macrophages/monocytes, eosinophils, neutrophils, mast cells and epithelial cells. The panel allowed for an efficient standardized gating strategy. Flow cytometry of immune cells in BAL fluid has proved useful in different clinical settings, as shown by interesting case examples.
Conclusions: The panel of 2 tubes 8-color combination of monoclonal antibodies offered an efficient method to perform differential cell counts in BAL fluid and provided phenotypic information not available from morphological evaluation.

PARALLEL SESSION 6: IMMUNOLOGY 2

PAR-06-01

NK RECEPTORS IN TUMOR IMMUNITY

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PD-1 is an immunological checkpoint that limits immune responses by delivering potent T cell inhibitory signals after interaction with its ligands expressed on tumor/virus-infected cells thus contributing to the immune escape mechanisms. Therapeutic PD-1 blockade was shown to accelerate tumor eradication with impressive clinical results. Little is known on the expression/function of PD-1 on human NK cells.
In the present study by multiparametric cytofluorimetric analysis we characterized a novel PD-1^{+} NK subset in normal donors as well as in cancer patients. In particular we show for the first time that PD-1 is expressed at high levels (PD-1bright) on an NK cell subset from approximately one fourth of normal individuals. This receptor is expressed by the $CD56^{dim}$ NK subset but not by $CD56^{bright}$ cells and is confined to a cell subset displaying the phenotypic features of fully mature NK cells being homogeneously $KIR^{+}NKG2A^{-}CD57^{+}$. Remarkably the frequency of this PD-1bright NK subset was increased in the ascites of a cohort of ovarian-carcinoma patients suggesting a possible enrichment of PD-1^{+} NK cells in tumor-associated environments. Functional analysis indicated that PD-1^{+} NK cells displayed reduced proliferative capability in response to cytokines as well as lower degranulation and cytokine production in response to tumor targets.

PAR-06-02

Probing T cell function

Steve Anderton
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CD4+ T cells are key orchestrators of immunity. They protect against infection and neoplasia, but their unwanted recognition of self, or innocuous foreign antigens forms the basis of autoimmune and allergic inflammation and transplant rejection. Understanding ways of manipulating desirable or unwanted T cell functions lies at the heart of the development of future immunotherapies. This talk will consider our recent data from disease models to highlight how flow cytometry can advance that understanding.

PAR-06-03

The proportion of Th22 cells in infants is reduced following maternal probiotic supplementation, and is associated with atopic dermatitis

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Introduction: In the randomized, controlled study Probiotics in the Prevention of allergy among Children in Trondheim (ProPACT), maternal probiotics supplementation reduced the incidence of atopic dermatitis (AD) in the offspring by 40%. The mechanism for this preventive effect is unclear. One possible explanation is an effect on T helper (Th) cells. The aim of this study was to examine whether the proportions of Th1, Th2, Th9, Th17, Th22 cells and regulatory T cells in three months old children were affected by maternal probiotic supplementation and whether the proportions of the Th subsets were associated with AD.
Methods: 415 pregnant women were randomized to ingest a combination of *Lactobacillus rhamnosus GG (LGG)*, *Bifidobacterium animalis subsp. lactis Bb-12 (Bb-12)*, and *Lactobacillus acidophilus La-5 (La-5)* or placebo. Their offspring were assessed for AD according to the UK Working Party's criteria during the first 2 years of life. Peripheral blood collected at 3 months of age and mononuclear cells were separated and cryopreserved until analysis. The proportions of Th subsets were analysed using flow cytometry FACSCanto II, (BD Bioscience, San Jose, Ca). Regulatory T cells were analysed in 140 samples using monoclonal antibodies against CD3, CD4, CD25, CD127 and FoxP3. Th subsets: Th1, Th2, Th9, Th17, Th22 in 77 samples were defined according to expression of

intracellular cytokines after *in vitro* stimulation using monoclonal antibodies against CD3, CD4, IFNγ, IL-4, IL-9, IL-17A and IL22. The study was approved by the Regional Committee for Medical Research Ethics in Central Norway. Participating women gave informed consent. The ProPACT trial is registered at ClinicalTrials.gov (NCT00159523).
Results: The proportion of Th22 cells was reduced in children from the probiotic group compared to the placebo group (median 0.038 vs 0.064, p=0.009). The proportion of Th22 cells among children with AD was increased compared to the children without AD (0.090 vs 0.044, p<0.001). The Th22 cell proportion increased with increasing severity of AD (p<0.001), and decreased with later debut age (p< 0.001). There were no significant differences in the proportions of other Th subsets or the Th1/Th2 ratio between the probiotics and placebo groups, nor were the other Th subsets associated with development of AD.
Conclusion: Perinatal maternal probiotics supplementation with a combination of LGG, Bb-12 and La-5 reduced the proportion of Th22 cells in 3 month old children. The proportion of Th22 cells was associated with severity of AD and debut age.

PAR-06-04

Distribution of different subsets of circulating monocytes in peripheral blood of healthy donors through life
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Introduction: Different subsets of peripheral blood (PB) monocytes have been described in humans, including classical (cMo; CD14+/CD16-), intermediate (iMo; CD14het/CD16het) and non-classical monocytes (ncMo; CD14het/CD16+). However, the distribution of these subsets has not been investigated through human life from newborns to elderly. Here, we analysed the distribution of 5 different subpopulations of monocytes in cord blood (CB) and PB specimens from a large cohort of individuals of different ages.
Methods: Eleven CB and 160 PB samples from healthy individuals were investigated for the distribution of cMo (CD62L+cMo and CD62L-cMo), iMo and ncMo (SLAN- and SLAN+ ncMo) according to age (from newborns to 92 year old adults) and sex. Samples were stained with a single 9-color tube including the following fluorochrome-conjugated monoclonal antibodies: CD14, CD16, CD45, CD62L, CD64, CD300e, anti-HLADR and anti-SLAN. Data was acquired in a LSR-FORTESSA X-20 flow cytometer (BD) and analysed with INFINICYT software (Cytognos).
Results: No evidence of sex-association in the whole series for the different subpopulations analysed (p>0.05) was observed, although slightly higher absolute numbers of cMo (p<0.05) were found in females *vs*. males in the 50-to-70y age-group. In contrast, we found marked differences in the distribution of circulating monocytes among the different age-groups (p<0.001). Thus, the greatest absolute counts of total monocytes was observed in CB, decreasing thereafter in parallel to age until 50y, such decrease being particularly pronounced during the first year of life. Of note, from 50y of age to the elderly (>80y), a slight but significant (p=0.02) recovery in the absolute count of total monocytes was observed. As expected, cMo showed the same pattern through life as described for total monocytes, as they represented the vast majority of monocytes in all the age-intervals; within this subset, CD62L+cMo were more represented (vs. CD62L-cMo) from newborns to puberty vs. adulthood. In turn, iMo and ncMo showed a similar behaviour to cMo, except for newborns, where the highest increase in the number of iMo and ncMo were observed vs. CB. Within ncMo, differences (p=0.001) in the relative distribution of SLAN- vs. SLAN+ PB monocytes were observed, since the former was more represented during the first 5y of life, while the later predominated thereafter.
Conclusions: Our results show that changes exist in the pool of circulating monocytes through human life, and provide a frame of reference as regards PB distribution of these cell subsets for further clinical studies and immune monitoring.

POSTER PRESENTATIONS

01

The role of CD49d expression in chronic lymphocytic leukemia cells

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Introduction: Chronic lymphocytic leukemia (CLL) is characterized by the progressive accumulation of $CD5^+$ $CD23^+$ B-cells in the peripheral blood, bone marrow and secondary lymphatic tissues. One of the strongest flow cytometry based prognostic factors is the high expression of CD49d molecule which predicts poor outcome. The CD49d is the α4 integrin subunit, associated with the CD29 (β1) molecule binds VCAM-1 and fibronectin. CD49d, together with CXCR4, plays key role in lymphocyte homing. It is well known that the progression of CLL is highly dependent of the tumor microenvironment. Our aim was to investigate the role of CD49d-VCAM-1 axis in the survival, cytoskeleton-remodelling and surface molecule expression of primary CLL cells after engagement by VCAM-1 and under conditions that mimic the bone marrow microenvironment.
Methods: Peripheral blood mononuclear cells were isolated from 30 CLL cases with different CD49d expression. Isolated CLL cells were cultured on VCAM-1 coated plates or in co-culture with bone marrow stromal cells (BMCSs). After 7 days the immunophenotype and apoptosis assessed by AnnexinV/propidium-iodide staining was measured by flow cytometry. Actin-reorganisation was investigated by phalloidin staining and visualized using confocal microscopy. Expression of the active conformation of CD49d/CD29 complex was detected by the high affinity conformation specific antibody (clone HUTS-21) and analysed by flow cytometry. The flow cytometric measurements were carried out using FACSCalibur (BD Biosciences) and analysed by CellQuest Pro software (BD Biosciences).
Results: There was no observed protection by VCAM-1 stimulation, while BMSCs significantly reduced the spontaneous apoptosis of CLL cells. The protective effect of the BMSCs was independent of the CD49d level, but showed correlation with CXCR4 expressed on CLL cells. VCAM-1 stimulation resulted in robust F-actin-formation by CLL cells with high CD49d expression. The CLL cells co-cultured with BMSCs have increased expression of CD5, CD49d, CD19, CD126 and decreased CXCR4; the VCAM-1 did not change the surface antigen levels. We detected only the low affinity conformation of the CD49d/CD29 on the CLL cell surface and triggering by various stimulus resulted just in moderate activation.
Conclusions: Our results suggest that the CD49d do not mediate direct survival signals, probably because it is present in inactive conformation. However through cytoskeleton-remodelling the CD49d may have important role in migration to protective lymphoid niches and adhesion to supportive accessory cells. Additionally, CXCR4 expression can indicate the sensitivity of neoplastic B cells to the favoring microenviromental signals.

02

Multisite Performance Evaluation Study of the BD OneFlow™ Plasma Cell Disorders (PCD) Panel

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Introduction: The BD OneFlow**™** solution for plasma cell disorders includes a completely standardized flow cytometry approach based on the Euroflow Consortium liquid multicolor antibody design. The BD OneFlow solution enables reproducible identification and discrimination of distinct cell populations by combining standardized assays, set-up reagents and protocols. The PCD antibody panel is composed of the BD OneFlow Plasma Cell Screening Tube (PCST) which helps differentiate normal plasma cell populations from those requiring follow-up and the BD OneFlow PCD tube, which helps differentiate abnormal from normal plasma cell populations. The BD OneFlow PCD classification tube characterizes the abnormal plasma cell population for positive identification of plasma cell disorders. The objective of this study is to compare the accuracy between the BD OneFlow™ PCD system and the equivalent EuroFlow liquid comparator system.
Methods: De-identified remnant human bone marrow specimens (n= 48) were collected at 2 study sites and tested in an unblinded manner within 26 hours of draw. Specimens were simultaneously stained with BD OneFlow reagents (PCD and PCST tubes) and liquid reagents specified by the Euroflow Consortium. Acquisition and analysis were performed on a BD FACSCanto II**™** instrument using standardized acquisition and analysis templates in BD FACSDiva**™** software. For qualitative endpoints, overall agreement, negative agreement and positive agreement along with their one-sided lower 95% confidence limits were calculated.

For accuracy quantitative endpoints (% positive plasma cell population), the slope, intercept and 95% confidence limits of the slope from a Deming regression were calculated for the BD OneFlow versus Euroflow methods.
Results: The BD OneFlow PCD system compared to the Euroflow system gave 100% (26 of 26) accurate results in classifying patients as having normal plasma cell populations and 100% (22 of 22) accurate results in identifying patients with a plasma cell disorder. Furthermore, the BD OneFlow PCD system correctly identified 100% of patients that had a plasma cell disorder based on test site clinical results.
Conclusions: The multicenter evaluation between the BD One Flow PCD system (PCST and PCD tubes) and the Euroflow liquid reagent system were fully concordant in identifying patients with abnormal plasma cell populations. Additionally, the clinical results for these patients confirmed a diagnosis of a plasma cell disorder. The BD OneFlow PCD panel is a fully standardized and validated system for aiding in the diagnosis of plasma cell disorders from bone marrow specimens. The BD OneFlow system is CE Marked to the European IVD Directive 98/79/EC.

03

Minimal residual disease detection by next generation flow-mrd in multiple myeloma patients
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Introduction: Detection of minimal residual disease (MRD) is a sensitive and rapid approach to evaluate treatment efficacy. Negativity of MRD predicts progression–free (PFS) and overall survival (OS) independent of categorical response assessment and patients biology in Multiple Myeloma (MM). Recently, two 8 colours tubes panel showed to be able to detect MRD with an increased sensitivity (10-5)(Next Generation Flow-MRD, NGF-MRD). NGF-MRD, applicable to virtually every patients in contrast to molecular approaches that required the development of a patient specific assays, became the preferred method to detect MRD. In MM patients, complete remission (CR) is prerequisite for long term PFS and OS, but little is known about long term remission patients (>5 years) that achieved at least a very good partial remission (VGPR).
The aim of our study is to identify the total plasma cell compartment (PCs) in bone marrow (BM) plus the percentages of both normal and neoplastic plasma cells, in long term remission patients using NGF-MRD approach.
Methods: In this study we analyzed 24 bone marrow (BM) samples of MM patients, 14 male, 10 female with the median age 58 (range 55-74 years), 13/24 were predefined as stringent CR (sCR), 4/24 as VGPR and 7/24 were tested while on treatment as group control. Samples were processed within 12h of collection, with a fix and perm protocol and staining using 2- tubes optimized 8-colours antibody panel (OneFlowTM PCST e PCD BD Biosciences).Acquisition was considered adequate when a significant PC population was detected or a minimum of 2 x 106 cells was acquired. PC populations were identified by gating with CD38 and CD138 positivity after exclude debris, doublets and lymphocytes. nPCs and aPCs are defined based upon a normal or abnormal pattern of expression of others antigens.
Results: In 2/24 cases the samples were not evaluable for the high peripheral blood contamination. Among 22 evaluable patients, only 9/22 showed an MRD+ status. In particular 2/12 in sCR, 2/4 in VGPR, 5/6 as group control. Interestingly, when evaluating the patients MRD+in sCR, the ratio PCn/PCs were 62,5% and 14%.
Conclusions: Preliminary results showed that NGF-MRD is applicable in 100% of patients studied but it requires good quality of samples. MRD negative status in the vast majority of long term CR patients could be a first step in defining Myeloma cure. Larger studies are needed to determine the MRD+ status in MM patients with clinical and economic important implications.

04

A single-center experience of flow cytometry investigations for paroxysmal nocturnal hemoglobinuria
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Introduction: Paroxysmal nocturnal hemoglobinuria (PNH) is a rare clonal hematological disorder, and its epidemiological aspects in Scandinavian countries have not been reported as yet. Karolinska University Hospital in Stockholm, Sweden, is a tertiary referral hospital where screening for PNH is performed, and serving a region of 2-million inhabitants. The aim of this study was to describe PNH-clones detected over a period of 6 years.

Methods: Flow cytometric analysis of GPI-anchored proteins is the method of choice for diagnosing and monitoring PNH. In Karolinska University Hospital, Swedac-accredited flow cytometric analyses for PNH clones are performed since 2012, with evaluation of FLAER-reagent, CD24, CD59, CD14, and CD48 expression in granulocytes and monocytes, and CD59 for analysis of erythrocytes. The lowest threshold for reporting a PNH-clone is 0.1% in any cell line. Study population included all samples referred for PNH-testing between 2009 and 2015. Samples with PNH clones were then analyzed according to the clone size (<1%, 1–9.9%, 10–49.9%, 50-100%).
Results: During the analyzed period, 303 PNH-analyses were performed in total of 251 patients. Of these, 41 (16%) patients had a PNH-clone, varying in size between 0.1–99%. Repeated tests were performed in 15 PNH-positive patients. The number of patients with first time detected PNH clone decreased from an average of eight persons per year during 2009−2013, to only a single patient per year during the 2014−2015. The study shows that mean incidence of newly detected PNH-clones of between 0.1 and 100% is 2.1 per year and million inhabitants in our region. Of all newly diagnosed PNH-clones, 38% were <1%, and having a PNH- clone of <10% was twice as common as >10%. PNH-clones were evenly distributed between age groups (age range in the study population: 6-84 years), and there was no gender difference.
Conclusions: Incidence of newly detected PNH clones between 0.1−100% have decreased in Stockholm region from 3.3/million/year to 0.4/million/year during the study period. This might reflect a true decline of PNH-clones incidence or variations in the frequency of diseases associated with PNH-clones; mainly bone marrow failure disorders such as myelodysplastic syndromes or aplastic anemia.

05

A rare hematological malignancy: blastic plasmacytoid dendritic cell tumor
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Introduction: blastic plasmacytoid dendritic cell tumor (BPDCN) is a rare neoplasm, whose exact incidence is unknown. It represents about 0.7% of primary cutaneous lymphomas. BPDCN is most common in the elderly with a modest male predominance. Besides the almost obligatory skin involvement, it usually infiltrates the bone marrow and has attendant leukemic dissemination (hematodermic tumor).
Methods: 79-year-old male presented with two violaceous bruise-like cutaneous lesions on the back, which were surgically excised. Subsequently he was admitted to the hematology department to investigate his hampered respiration and hepatalgia. The examination included pulmonary scintigraphy, abdominal ultrasound and detailed laboratory investigations (CBC, clinical chemistry, immunophenotyping, cytogenetics, FISH and histology).
Results: abnormal cells originating from the same pathological population could be identified in the peripheral blood, bone marrow and in the skin sections. The smears and the dermis contained agranular mononuclear, sometimes vacuolated plasmacytoid tumor cells, which showed CD45+, CD123+, CD56+, HLA-DR+, TCL1+, DAP12+ and dim CD4+ staining with immunohistochemistry and flow cytometry. Cytogenetics detected hypodiploid metaphases (3/20) with random chromosome losses, while the FISH confirmed heterozygous deletion of the 13q14 region (DLEU) and the 12p13 region (ETV6), besides homozygous deletion of the 9p21 region (CDKN2A/B).
Conclusions: Despite CD4 was underexpressed compared to literature data, a congruent diagnosis of BPDCN was settled based on the above findings. Since the disease tends to be aggressive, early diagnosis is crucial, which can be supported by the specific CD123/CD56/CD4 triple positive immunophenotype and the presence of certain antigens as BDCA2, TCL1 and CD2AP. Guidelines suggest to start the treatment with hyper-CVAD, but the patient did not tolerate it, and departed at the 14th day of therapy because of pneumonia and heart failure.

06 (Selected Best Poster Abstract Presentation)

A cost-effective, high sensitivity 10-color single-tube flow-cytometry (FC) based B-cell precursor acute lymphoblastic leukemia (BCPALL) minimal residual disease (MRD) assay
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Introduction: Minimal residual disease (MRD) monitoring is widely used for treatment-effectiveness and risk-stratification of ALL. Hence, the methodology for MRD assessment needs to be fast, highly sensitive, affordable and most importantly, widely applicable. Studies have shown that flow-cytometry-based MRD (FC-MRD) technique

cannot reach beyond the 0.01% sensitivity and PCR-based MRD-technique is more sensitive. We present a study of a cost-effective high-sensitivity 10-color single tube FC-MRD assay.
Methods: We studied 102 cases of BCPALL in Hematopathology-Laboratory, Tata Memorial Centre, Mumbai. FC-immunophenotyping was performed on Navios flow-cytometer using bulk-lysis-and-stain method and data was analyzed with Kaluza-software. MRD was monitored in 163 bone-marrow samples at post-induction (PI, day 29-35), post-consolidation (PC, day-90) and subsequent follow-up time-points (SFU) using 10-color FC-MRD-assay. 10-color MRD-panel included CD123, CD20, CD58, CD86, CD25, CD19, CD10, CD34, CD45 and CD38. An additional four-color nuclear-dye(SYTO-13)-tube was used for calculation of MRD in total nucleated-cells. Samples with a cluster of ≥20 events expressing ≥2 leukemia-associated immunophenotypes (LAIPs) were called MRD-positive.
Results: Of 102 cases studied, 67 were childhood-BCPALL (0-14 years) and 35 were adult-BCPALL (>14-64 years). In FC-MRD assay, high number of events were acquired with median-events 2113000 (range, 506000 to 3010000). Of 163, MRD was positive in 84 (55%) samples (median, 0.135% and range, 0.0007%-47.5%). MRD-positive samples with MRD levels <0.001%, 0.001- <0.01%, 0.01- <0.1%, 0.1- <1.0%, 1.0- <10% and >10% were respectively 2.4%, 7.1%, 34.5%, 27.4%, 14.3%, and 14.3%. Of 163 MRD samples, PI-MRD were 102 (62.6%), PC-MRD were 38 (23.9%) and SFU were 22 (13.5%). Of 102 diagnostic-samples, <2-LAIPs were seen in only 1% and >6-LAIPS in 2% (median-LAIPs, 4 & range, 1-8). In MRD samples, four had <2LAIP (2.4%) and labelled as “suspicious”. Of 84 MRD-positive, samples with 2-LAIPS were only 7.1%. Furthermore, in 10 samples with MRD-positive ≤0.01% and >1.5-million events, the results were compared between time-gated initial 500000-events, 1000000 events and all acquired-events. Amongst them, five samples found negative in initial 500000-events and three in initial 1000000-events (since number of MRD-events were <20-events) highlighting importance of acquisition of >1.5-million cells to increase the sensitivity of FC-MRD-assay. Cost-per-assay was 25 USD. **Conclusion:** We established a cost-effective, 10-color, single-tube FC-MRD-assay with high sensitivity of 1-in-10^5 MRD detection and applicability in >97% samples. Hence, this assay could be highly useful in resource-limited settings. Our study also showed that the acquisition of less than one-million events can reduce the sensitivity of FC-MRD-assay.

07

Dynamics of PNH-clon in aplastic anemia patients during immunosuppressive therapy

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Introduction: The frequency of PNH-clone detection in Aplastic Anemia patients at the early stages of the diagnostic increased up to 79%. Nevertheless, PNH clone detection is interesting not only from the point of view of progression into hemolytic form of PNH (from 2 to 19% AA patients). The presence of PNH clone in AA patients can be accompanied with undetected hematopoiesis disorders, with follow possibility of manifestation under proliferative stress. Therefore PNH clone has to be monitored in AA patients during all the time of treatment.
Methods: This study is a prospective research when the analysis of PNH clone size was made in 47 AA patients during immunosuppressive therapy (IST). Among them initially PNH clone was detected in 59,6% of patients. The median of time observation was 27 (9-48) months. Depending on the PNH clone size on granulocytes all patients were divided into 4 groups: the 1st group - from 0,01% to 0,99% (n=11); the 2d group - from 1% to 9,99% (n= 8); the 3d group - from 10% to 49,9% (n= 4); the 4 group - from 50% and higher (n=5).
Results: The variety of trends of the PNH clone dynamic was detected. Three patients from the 1st group showed the growing of PNH clone size from the minor clone (less than 1%) to 3,55%. Moreover, 1 patient during 12 months observation showed a full elimination of the PNH clone. A visible one-way PNH clone dynamic was detected in patients from 3d group: during 3 month of observation, with simultaneous disease remission, the median rate of PNH clone size in group was going down from 22,9% (18,39-24,77%) to 5,6% (1,5-6,7%). The PNH clone size in patients from both 2nd and 4th group was stable. The evaluation of the hemolytic PNH was shown in all patients from the 4th group, i.e. by 18% of AA patients with initially detected PNH clone. During our observation 37% of all AA patients without initially detected PNH clone have shown the appearance and persistence of PNH clone (the Median - 0,34% on granulocytes (0,1-6,2%.)
Conclusion: According to this study the change of the PNH clone size or its appearance during response on IST and most likely depends on the grow advantage in the process of a normal (GPI-positive) or clonal (GPI-negative) hematopoiesis. Evaluation and development of molecular diagnostic methods and follow up patients in dynamic will help to get exact answers.

08

A single-platform pattern-recognition analysis strategy for flow cytometry minimal residual disease detection in B-cell precursor lymphoblastic leukaemia

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Introduction: An accurate detection of minimal residual disease (MRD) in B-ALL is important for refining risk-adapted therapy and detecting MRD reoccurrence. Detecting MRD by flow cytometry (Flow-MRD) requires leukaemic cells to be distinguished from normal B-cell precursors (haematogones,HG). Assessing concordance between Flow-MRD and the gold-standard Molecular (Mol-MRD) analysis will verify the use of a newly developed Flow-MRD analysis strategy.
Methods: MRD was measured on 141 bone marrow samples from 69 B-ALL patients (paediatric and adult). Flow-MRD was tested using a validated single 8-colour tube (TDT-FITC, CD10-PE, CD34-PerCPCy5.5, CD19-PE-Cy7, CD33-APC, CD38-APCH7, CD20-BDHorizonV450, CD45-BDHorizonV500) by a standard membrane/intracytoplasmic staining method, analysed with FACSDiva software and FACSCantoII cytometer. Control samples (n=23) were analysed to determine the expression pattern of HG, mature-B-cells and plasma cells. Between 200,000 and 500,000 events were acquired (sensitivity 1:10^4). The analysis strategy excluded debris/doublets, gated mononuclear cells followed by CD19pos/CD10pos and CD19pos/CD10neg gates. Populations were analysed separately to distinguish CD10-positive residual blasts from HG and demonstrate the phenotype of CD10-negative blasts after excluding mature-B-cells and plasma cells. As immunophenotypic shift may occur, analysis is not restricted to blasts with the same diagnostic phenotype. It is based on the immunophenotypic profile of all blasts/HG, and is particularly useful for heterogeneous blast populations. The Flow-MRD was compared to Mol-MRD for Ig-gene rearrangements (IG-QPCR) or fusion transcripts (RQ-PCR). The cut-off values for IG-QPCR as defined by UKALL2011 were 0.005% at day29 and 0.05% at week14.
Results: Results were considered concordant when positive by Flow-MRD (>0.01%) and above Mol-MRD cut-off or negative by Flow-MRD and negative or below Mol-MRD cut-off. The remaining results were considered discordant. Concordance within the group tested by IG-QPCR (n=48) was 89.6%. Within the RQ-PCR groups, the Ph-negative B-ALL group (n=17) showed 82.4% concordance and the Ph-positive group (n=76) - 71.1%. Discordant results within the groups were respectively 10.4%, 17.6%, 28.9% - all Flow-MRD-negative and Mol-MRD-positive.
Conclusions: The newly developed analysis strategy clearly indicates success in detecting B-ALL MRD with sensitivity of 1:10^4. Good concordance was achieved in the group tested by IG-QPCR and the Ph-negative group. The discrepancies found could be due to sample quality, different sensitivities and principles of the two techniques (measuring cells vs. DNA). All positive Flow-MRD results were confirmed by Mol-MRD and can therefore serve as a therapy guide whilst Mol-MRD is pending. Negative Flow-MRD findings should be considered correct within the limits of the sensitivity of the method but need PCR verification.

09

Evaluation of regulatory T- cells' alterations in Multiple Myeloma patients receiving lenalidomide or bortezomib-based regimens

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Introduction: Immune dysfunction is an important feature of Multiple Myeloma (MM) and has been associated with reduced survival. Studies have shown that T regulatory cells (Tregs) implicated in immune surveillance are expanded in tumors, including MM. Data regarding alterations of Tregs during therapy with Novel Agents (NA) i.e. bortezomib and lenalidomide, are limited. Our aim was to explore possible alterations of Tregs and lymphocyte subpopulations (T4, T8, B, NK, NKL), as well as changes in the levels of cytokines related to Tregs function and MM biology (IL-6, IL-2, IL-17, TGF-β) during treatment with NA and to seek for correlations with disease characteristics and response parameters.

Methods: We evaluated 29 patients with symptomatic MM at diagnosis or relapse (M/F: 15/14, median age: 61 years, range: 39-77). Eleven patients received bortezomib-dexamethasone (BD) (group A) and 18 patients received lenalidomide-dexamethasone (Rd) (group B). The median number of previous treatment lines was 2 (0-3). The detection of Tregs and lymphocyte subpopulations was performed in peripheral blood samples using polyparametric flow cytometry analysis and the appropriate isotypic controls. The cytokines were measured in serum samples using the enzyme-linked immunosorbent assay ELISA. The statistical analysis was performed with the appropriate methods; $p<0.05$ was considered statistically significant.
Results: In group A, no significant alterations of Tregs%, lymphocyte subpopulations or cytokines were observed during treatment. In group B, there was a significant reduction of Tregs % ($p<0.001$) and this was more profound in those who achieved ≥vgPR ($p=0.04$). No alterations regarding lymphocyte subpopulations or cytokines were observed during treatment with NA in either group of patients. Patients had significantly higher median Tregs% compared to HV ($p<0.001$). There were no significant correlations between disease characteristics and Tregs in either group of patients. In the Cox regression analysis, Tregs% did not correlate with progression-free survival (PFS).
Conclusions: Herein, we have demonstrated that Tregs% were significantly reduced after treatment with Rd especially in patients with ≥vgPR, suggesting a possible relation of immune surveillance with quality of response. However, PFS was not affected in the current study. Bortezomib-based treatment had no impact on Tregs number. Patients with myeloma had higher Tregs% compared to HV, confirming the implication of immune impairment in the biology of this disease. No relation between Tregs and disease characteristics was observed in this study, nor between Tregs and relative cytokines, indicating that immune mechanisms underlying MM remain unexplored.

10 (Selected Best Poster Abstract Presentation)

High expression of embryonic transcription factor OCT¾ associated with differentiation anomalies in acute myeloid leukemia and myelodysplastic syndromes

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Introduction: Acute Myeloid Leukemia (AML) and high-grade myelodysplastic syndromes (MDS) are characterized by the expansion and resistance to apoptosis of poorly differentiated myeloid cells. Long term propagation of the disease is mainly due to a subset of cells termed leukemic stem cells (LSC), which reacquire self renewing properties and give rise to the leukemic clone. Several studies have suggested that LSC mostly belong to the $CD34^+CD38^-$ compartment and represent the malignant counterpart of normal hematopoietic stem or progenitor cells (HSC). Self-renewal and lack of differentiation are also features of embryonic stem cells, characterized by the expression of specific genes including those known as "Yamanaka's factors", including the transcription factor OCT¾. A few reports show an abnormal expression of OCT¾ in solid tumors in which it induces pluripotency and suppresses differentiation, but no study is available in AML.
We investigated the potential role OCT¾ in leukemogenesis by evaluating its expression in stem cells from AML and MDS patients compared to normal HSC subsets ($CD34^+CD38^-$ and $CD34^+CD38^+$).
Methods: We studied 139 AML and 42 MDS newly diagnosed cases by Multicolor Flow Cytometry (MFC) and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). We also evaluated in HL60 leukemic line its potential involvement in myeloid differentiation by inhibiting its expression by short hairpin RNA (shRNA).
Results: In MCF, we observed an up-regulation of OCT¾ in AML cells as compared to normal cells (Mean of fluorescence 4.5 vs 3.2, $p<0.05$, cycle threshold 26 vs 37, $p<0.05$). This expression was higher in $CD34^+CD38^-$ than in $CD34^+CD38^+$ subsets in normal as well as in leukemic marrows. There was no evidence of preferential association with cytogenetic or molecular characteristics of AML. Furthermore, an over-expression of OCT¾ was observed in MDS, more specifically in forms with excess of blasts.
We also evaluated the prognostic value of OCT¾ expression in the 103 AML patients who received an intensive treatment. The rate of complete remission was not influenced by the OCT¾ level. There was a trend ($p=0.06$) for better overall and leukemia-free survival for patients with high OCT¾ expression.
Finally, HL60 differentiation with retinoic acid induced a decrease of OCT¾ expression. Inhibition experiments by shRNA showed a strong downregulation of OCT¾ and an arrest of proliferation associated with myeloid differentiation.
Conclusion: These results suggest that aberrant expression of OCT¾ in AML and MDS is associated with differentiation blockade.

11

Seven-year-follow-up of low-count monoclonal B-cell lymphocytosis: B-cell clones persist at increased counts without evidence for clinical progression

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Introduction: Low-count monoclonal B-cell lymphocytosis (MBL^{lo}) –defined as the presence of $<0.5x10^9$ clonal B cells in the peripheral blood (PB) of otherwise healthy subjects – is a common finding in the general population. The vast majority of MBL^{lo} clones show a CLL (chronic lymphocytic leukemia)-like phenotype, their clinical and biological significance remaining to be elucidated. Thus, they might represent either a normal physiological B-cell population (i.e the CLL cell counterpart) or the earliest stages of CLL (i.e a preleukemic condition); limited information exists about the outcome of MBL^{lo} overtime. Here, we aimed evaluated the outcome of a series of 55 MBL^{lo} subjects after a median follow-up of 7 years.
Methods: Eighty of the 639 initial screened participants (>40y) had at recruitment at least one detectable clonal (minimum number of clustered events required to define an abnormal B-cell population was of 25 cells) CLL-like B-cell population in PB (12.5%) by high-sensitivity 8-color flow cytometry; since then, most of these MBL^{lo} subjects (n=55) have been followed for a period of between 5 and 8 years (median of 84 months; range 60-95). A change in size of clonal B-cell populations overtime was defined when such count was outside the confidence intervals of 99% obtained by bootstrapping statistical test.
Results: Overall, at baseline time we identified 74 CLL-like MBL^{lo} clones (median size: 0.44 cells/µl range: 0.027-66 cells/µl) in the 55 subjects analyzed; 35 clones were from monoclonal cases and 39 from multiclonal individuals (n=20). In all subjects, B-cell clones persisted at reevaluation with an identical phenotypic profile as initially observed. However, the number of clones detected –monoclonal *vs*. biclonal– varied in 3 cases (5.5%). Regarding the clone size, a significantly higher counts were observed in 55/74 CLL-like B-cell populations (74%) with a 3-fold increase (range: 1.3-73 fold); meanwhile 10 clones (14%) showed a smaller size (median decrease: 3-fold, range: 1.01-5.2) and 9 (12%) remained stable. Of note, subjects showed normal lymphocyte counts at reevaluation, and none of the cases but one evolved to MBL^{hi}.
Conclusions: Our results suggest that MBL^{lo} is not a transient condition; since all clones detected at diagnosis were systematically present also at follow-up usually at slightly increased counts. Despite this, the rate of progression to MBLhi and CLL appears to be extremely rare. Further research is required to better understand the biological parameters associated to the expansion of MBL^{lo} B-cell clones and elucidate their relationship with an overt CLL.

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Utilization of the ClearLLab lymphoid screening tube for investigation of hematolymphoid neoplasias by flow cytometry

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Introduction: Flow cytometry immunophenotyping is an integral part of diagnostics for hematologic malignancies. The ClearLLab Lymphoid Screening (ClearLLab LS*, Beckman Coulter, Miami, FL) tube contains the following markers: CD8-FITC, Kappa-FITC, CD4-PE, Lambda-PE, CD19-ECD, CD56-PE-Cy5.5, CD10-PE-Cy7, CD34-APC, CD5-APC-A700, CD20-APC-A750, CD3-PB and CD45-KO in a dried format and should prove useful as a screening tube for hematolymphoid malignancies.
Methods: A blinded comparison between individual laboratory developed tests (LDTs) and ClearLLab LS was performed to 1) identify pathologic cell populations and 2) exclude hematological malignancy. 167 diagnostic samples (61 bone marrow, 22 lymph node and 84 whole blood samples) were assessed across three 3 North American/European laboratories with their respective LDTs and in parallel by the single ClearLLab LS reagent panel.

Results: All of the 80 samples with no hematological malignancy as analyzed by LDT were also negative for hematological malignancy by ClearLLab LS (100% agreement).
Of 87 samples with a hematological malignancy and an abnormal population within the sample tested by LDT, 83 were positive by ClearLLab LS resulting in a 95.4% agreement compared to LDT. Of the 4 cases which were not detected positive by ClearLLab LS, 2 were plasma cell neoplasms with minimally involved bone marrow samples. Two mature T cell malignancies with loss of CD7 were also not detected by the screening tube. The presence of CD34 in the tube allowed the detection of blast populations in several myeloid neoplasms that were tested.
Discussion: The ClearLLab LS screening tube showed excellent agreement with LDTs developed by three international laboratories. Mature T cell malignancies in general require a multiple disciplinary diagnostic approach and cannot be ruled out by flow cytometry only. Thus, the absence of CD7 from the ClearLLab LS may be considered acceptable. Regarding plasma cell dyscrasias, low level plasma cell infiltration are diagnostically challenging and particularly so by ClearLLab LS, however, morphology and more specific laboratory tests are unlikely to result in a missed diagnosis.
**ClearLLab LS is in development, pending achievement of CE compliance; not available for in vitro diagnostic use.*

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Interest of Raman Microspectroscopy in the Diagnosis and Prognosis of B-Chronic Lymphocytic Leukemia
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Introduction: The current strategy of B-Chronic Lymphocytic Leukemia (CLL) diagnosis is mainly based on the immunophenotyping by FC. This technique is operator dependent, time consuming (approximately 60 minutes for the diagnosis of CLL) and requires specific knowledge's and instrumentation. Since immunophenotypeis not available in many laboratories, the diagnosis is often delayed and this may impact the patient clinical outcome and always the healthcare costs.
Methods: Raman Microspectroscopy (RM) is a non-destructive method for the observation and characterization of the molecular composition and structure of materials and cells.
It has recently been showed that RM is able to discriminate accurately benign from malignant lymphocytes in opposition to haematological analyzers. The aim of our research is first to evaluate the interest of the RM for the diagnosis of B-Cell Chronic Lymphocytic Leukemia (CLL) and the definition of easily accessible prognosis parameters and second, to evaluate the practicability of RM in a routine hematology laboratory.
Results: The specificity and sensitivity of RM are 84.6% and 90.9% respectively when we targeted on region of interest of spectrum. It allows us to make the difference between RM spectra from Healthy B-Lymphocytes (HBL) and CLL B-Lymphocytes (CBL). These early results show that visual analysis and comparison of RM spectrum are not easy and requires multi-dimensional and mathematical analysis. For those purposes, Classifier and Multi-Parameters Spectra Analyzers (CMPSA) have been built to make automatically the difference between HBL and CBL. To create the CMPSA, HBL, CBL, T and NK lymphocytes have been sorted by Flow Cytometry (FC) to analyze purified cells from healthy subjects and CLL patients.
Conclusions: RM offers a lot of advantages for laboratory including automation, short time of acquisition (<30min), operator independent's interpretation and informations about molecular structure of the nucleus that both FC and morphology are not able to give. Today, no predictive parameters for the clinical outcome of the disease have been found but immunoglobulin rearrangement of CBL could be detected by RM keeping the hope for RM predictive parameters. Actually, one hundred B cells by sample are inspected by RM. Decreasing the number of studied cells could have a significant impact on the quickness and the comfort of the CLL (differential) diagnosis. This could be done in a near future by using quantitative phase-contrast microscopy.

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B lymphoproliferative disorder with subsequent development of plasma cell leukaemia

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Introduction: The development of plasma cell leukaemia (PCL) in a patient with low stage B lymphoproliferative disorder (LPD) has not been reported. The combined use of laboratory methods in addition to close clinical surveillance was essential for timely diagnosis.Methods:We describe clinical and laboratory findings in a 69-year old male patient who developed PCL 8 years after initial diagnosis of a low stage B LPD and three years after treatment. Immunophenotypic, immunohistochemical and morphologic evaluation of peripheral blood (PB) and bone marrow (BM) were performed at several time points from diagnosis to the appearance of new symptoms.
Results: Presenting features included lymphocytosis in routine testing, with normal concentration of creatinine and lactate dehydrogenase. Initial PB flow cytometric (FC) immunophenotyping supported the presence of kappa restricted B cells with non-CLL phenotype (CD5+, CD20+, CD23-, CD43-, CD79b+, FMC7+ B cells,3,500/μl) with respective findings four years later. Cytogenetic analysis detected trisomy 2, 3, 12 and 18. In accordance to PB, BM biopsy and immunohistochemistry (IHC) revealed infiltration by a low grade mature B cell non-Hodgkin lymphoma (cyclin D1- B cells, 60% of BM cells). Inspite of minor lymphadenopathy, deteriorating splenomegaly with concommitant thrombocytopenia led to treatment with cyclophosphamide, vincristine, and prednisolone and, after spleen size reduction, with fludarabine, cyclophosphamide and rituximab. Three years later without treatment, the patient presented with weakness, lameness and bone pain. Imaging exams revealed multiple osteolytic lesions, suggesting plasma cell myeloma. BM biopsy and IHC revealed 80% infiltration by lymphoid appearing cyclin D1+ kappa restricted plasma cells (PCs), whereas there was no evidence of lymphoma infiltration. FC immunophenotyping of BM aspirate confirmed the presence of CD20-, CD56-, CD45-, CD19-, CD117-, intracellular kappa clonal PCs (54% of nucleated) and persistence of kappa restricted B cells with mostly similar to initial diagnosis phenotype (11% of nucleated). Moreover, PB FC immunophenotyping revealed abnormal circulating PCs (22.7% of nucleated, 2,865/μl) and increased B cells (1,238/μl), establishing the diagnosis of concurrent PCL and B LPD. Serum electrophoresis and immunofixation detected presence of IgA-kappa and free kappa light chain monoclonal protein, despite low concentration of serum immunoglobulins. The patient has been treated with myeloma-specific therapy.
Conclusions: To our knowledge, this case is the first reported of PCL development in a patient with non-Hodkin B LPD. Although molecular analysis was not feasible, the discordant expression of cyclin D1 by clonal B lymphocytes and PCs imply that they may originate from separate B cell clones.

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Myeloid derived suppressor cells in CLL patients

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Introduction: Myeloid derived suppressor cells – MDSCs are a phenotypically and functionally heterogeneous population of immature cells belonging both to the monocytic (mono-MDSCs) and the granulocytic (gran-MDSCs) line. They play a potent suppressive effect on adaptive and non adaptive immune response in patients with cancer, and chronic and acute inflammation. The purpose of this study was the determination of MDSCs in patients with CLL and their correlation with the number of neoplastic cells.
Methods: 19 PB samples of patients with CLL, 11 of patients with MBL and 10 samples of healthy subjects (HS) were studied with multiparametric flow cytometry. The combination of HLADR-FITC/ CD64-PE/ CD45-ECD/ CD14-PC5/ CD3-PC7/ CD19-PC7 was used for the determination of mono-MDSCs as a percentage of monocytic cells and the combination of CD15-FITC/ CD11b-PC5/ CD3-ECD/ CD19-ECD/ HLADR-PE/ CD33-PC7/ CD45- AlexaFluor750 was used for the determination of gran – MDSCs as a percentage of total PB cells on a Navios BC flow cytometer.
Results: A statistically significant increase of mono-MDSCs was found among patients with CLL, MBL and HS (4,99±1,67 vs 3,66±0,93 vs 1,92±1,14, ANOVA p<0,0001). This statistically significant increase concerns both the difference between CLL and HS (p<0,001), and the difference between MBL and HS (p=0,001) as well as between CLL and MBL (p=0,023). Nevertheless no statistically significant correlation was found between the CD19+CD5+CD23+ B lymphocytes absolute number and mono-MDSCs percentage. An increase of gran-MDSCs percentage was found between the three groups that did not reach statistical significance.

Conclusions: The flow cytometric study of mono- and gran-MDSCs is possible although it needs standardization. The determination of these cells possibly contributes to CLL patients prognosis as they seem to increase significantly their percentage. Further study and comparison with other prognostic markers is needed.

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Reference cell populations of clinical samples in antibody validation process at a flow cytometry laboratory

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Introduction: Flow cytometry clinical laboratories are responsible for evaluating antibody performances before they are used in clinical samples. In order to verify an antibody's adequacy technicians must know the antigen distribution and the positivity patterns expected in the test samples. Although the former is presented by most manufacturers in the data sheet, the choice of which kind of sample and cell population is used for performance evaluation is a matter of laboratory technical decision. Here we present which samples and populations, along with their positivity pattern, are used in our laboratory to validate most of the antibodies used to perform the diagnosis of haematological neoplasms.
Methods: The analysis were performed at the Flow Cytometry Laboratory (College of American Pathologists accredited) of Hospital das Clínicas da Faculdade de Medicina da Universidade de São Paulo from January to December of 2015. Fifty-six antibodies used for the diagnosis of haematological neoplasms were tested on bone marrow or peripheral blood samples, following the manufacturers' recommendation.
Results: The brightest cell population was used as the reference to evaluate the performance of each antibody, even in cases where the antigen is present in more than one cell population (*e*.g.: for CD4, T-lymphocytes were used, although the antigen is also present in monocytes). Regarding the type of sample used, 88% of all antibodies were validated using an appropriate reference cell population found in normal peripheral blood or bone marrow samples, while 13% required a pathological sample because the antigens are absent or not so abundant in normal samples, these are: CD1a, CD34, CD103, CD117, CD123, CD138 and TdT.
Conclusions: The manufactures' data sheet usually describes a broad spectrum of positivity in several cell linages. We show how our laboratory uses target cell populations to validate antibodies that are routinely used for the diagnosis of haematological neoplasms. Most antibodies could be validated using normal samples that are easily accessible to clinical laboratories. However, reference populations that are only found in pathological samples can be a problem if it requires a sample of a rare disease that is not readily available at the time of the validation.

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Flow cytometric detection of CLL transformation to DLBCL: case report

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Introduction: Chronic lymphocytic leukemia (CLL) is the most common type of leukemia found in adults. CLL may transform into a fast growing diffuse large B cell Lymphoma (DLBCL). We present a case of a 57 year old female patient with a progressive CLL which has been known since 1989.
Methods: 5 color flow cytometry (FC) (Beckman Coluter FC500) was done using specific panel combinations for the detection of B cell lymphomas. For the analysis, appropriate software for cell population gating was used. Additionally, fluorescent "in situ" hybridisation (FISH) and chromosomal banding analysis were done.
Results: A typical CLL (B cell population without surface light chain restriction, aberrant positive expression of CD5, positive for CD19, CD23, dim CD20 and negative for FMC7, CD79b, CD22) was confirmed in patient by FC in 2013 and repeated in February 2016 due to progressive disease. Cytogenetic analysis was also done showing a normal femal karyotype. In 2016 immunophenotypisation of bone marrow aspirate showed two distinct B cell lymphomas. The major B cell population (18%) expressed an immunophenotype, appart for a negative CD20, typical for CLL (no surface light chain restriction, aberrant positive expression of CD5, positive for CD19, CD23, CD38 and negative for FMC7, CD22 and CD79b). The second population (12%) with a higher forward scatter expressed surface kappa light chain restriction, positive for CD5, CD22, CD79b, CD30, CD38 and bright CD19. We concluded that the second population, with the exception of negative CD10, corresponds to DLBCL. DLBCL retained a CLL immunophenotype, which indicates that the primary CLL clone transformed to DLBCL. Cytogenetics also found two different clones. The one with deletion of gene ATM (11q) in 15% of cells, corresponding to typical CLL clone, and a hyperdiploid clone with numerous chromosomal aberrations, corresponding to DLBCL clone.

Conclusions: In previously known patients with typical CLL the second malignant B-cell population can be easily overlooked. Especially in cases where due to different scattering characteristics population is located in unusual position in histograms. In our case these were big DLBCL cells arising through CLL Richter transformation.

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Usefulness of flow cytometry for the diagnostics of myelodysplastic syndromes in Vilnius University Hospital

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Introduction: Diagnosis of myelodysplastic syndromes (MDS) is based on complete blood count (CBC), morphologic features of hematopoietic cells and cytogenetic abnormalities. Aproximately half of MDS cases are cytogenetically "silent", results of morphological analysis are might be inconclusive. Flow cytometric analysis could aids in detecting blast percentage, myeloid blast phenotype and granulocyte, monocyte and erythrokaryocyte maturation alterations.
Methods: 56 adult patients admitted to Vilnius University Hospital "Santariskiu Klinikos" with MDS in suspicion were included in the study (29 males and 27 females). CBC analysis was performed on Sysmex XE-5000 hematology analyzer. Cytological, histological and cytogenetic tests were carried out for bone marrow samples. Based on the results patients were attributed to MDS or non-MDS groups. Bone marrow samples were analyzed on FACSCantoII flow cytometer 6 color MDS marker panel. Percentage of hematopoietic precursors (myeloid blasts and hematogones), the phenotype of myeloid blasts, monocytes, granulocytes and erythrokaryocytes, deviations from normal maturation patterns of hematopoietic cells (SSC/CD11b/CD13/CD16, CD64/CD10 for neutrophils, CD14/CD36 for monocytes and CD71/CD235a for erythrokaryocytes) were compared between MDS and non-MDS groups. Every deviation was equal to 1 point to total flow MDS score.
Results: Median MDS score was 6.47 (± 1.94) versus 2.77 (± 2.29) in non-MDS group. Score of four gave best discrimination between MDS and non-MDS with specificity of 73.3 % and sensitivity of 80.0 %.
Average amount of blasts of MDS patients was 10.27 (±8.48) % versus 2.11 (±1.92) % ($p<0.01$) in non-MDS patients. There were several phenotypic aberrations in blast population of MDS patients: hypoexpression (CD13 and CD33), crosslineage expression (CD7 and CD56), coexpression (CD56 in monocytes) in MDS vesrus non-MDS population. However, no single flow cytometric parameter differentiated MDS positive and negative groups with statistical significance.
Markers that differentiated MDS positive and negative cases the best were:
(1) reduced count of hematogones ($p=0.03$),
(2) reduced SSC in granulocyte population ($p=0.10$),
(3) changes in granulocyte CD10/CD64 and CD11b/CD13/CD16 expression pattern ($p=0.03$ and $p<0.01$ respectively), and
(4) changes in erythrokaryocyte CD71/CD235a expression pattern ($p=0.05$).
Conclusions: Although the final diagnosis of MDS still depends on morphologic and cytogenetic findings, flow cytometry can be useful to identify patients with higher risk of MDS for further monitoring. Flow cytometric MDS assays still require improvement, particularly standardization of marker panels, comparison of MDS patients to normal controls, elderly, post chemotherapy or megaloblastic anemia patients.

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Unusual locations of extramedullary relapse in multiple myeloma diagnosed by multiparametric flow cytometry and the role of CD56

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Introduction: Extramedullary relapse constitutes an uncommon manifestation of multiple myeloma, but central nervous system involvement as the only manifestation of relapse, as well as abdominal plasmacytomas and ascites seem even less common. Multiparametric flow cytometry has recently been shown to be an essential tool for diagnosis and minimal residual disease detection in multiple myeloma patients. However, data in the literature

regarding cerebrospinal and ascitic fluid examination by flow cytometry in myeloma patients are limited, since most of the cases were diagnosed by cytologic assays.
Methods: We present four patients with plasma cell neoplasia and extramedullary relapse. Three patients with multiple myeloma and one patient with de novo plasma cell leukemia achieved complete remission after autologous stem cell transplantation. Few months later, while being in systemic remission, two of the patients with multiple myeloma as well as the patient with plasma cell leukemia presented with neurological manifestations. The third myeloma patient presented with abdominal plasmacytomas and ascites. Cerebrospinal and ascitic fluid samples were studied by 5 and 6-color combinations of monoclonal antibodies.
Results: Plasma cells were identified as CD38(+)/CD138(+) events and were further characterized by immunophenotypic aberrations and light chain restriction. Regarding CD56 expression, plasma cells infiltrating cerebrospinal fluid samples were CD56 positive, while those in ascitic fluid, CD56 negative. Plasma cells were CD19 negative in all samples.
Conclusion: The role of CD56 (neural adhesion molecule) in favoring extramedullary localization of multiple myeloma is controversial. Downregulation of CD56 was considered essential in the pathogenesis of malignant plasma cells escape from bone marrow microenvironment. However, more recent reports are not in agreement with such hypothesis, as CD56 was positive on myeloma plasma cells in cases of central nervous system involvement. We confirm that flow cytometry is a useful tool providing rapid diagnosis of extramedullary multiple myeloma relapse.

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CD5+ myeloid and plasmacytoid dendritic subpopulations with overlapping features. Evidence in favor of plasticity?

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Introduction: During the assessment of minimal residual disease (MRD) in a case of acute T lymphoblastic leukemia (T-ALL), there was observed a suspicious population of CD5+, surface CD3-, CD7- and CD45 low cells. Further investigation revealed that they were not MRD, but CD5 positive myeloid (mDC) and plasmacytoid (pDC) dendritic cells. Their further phenotypic evaluation unexpectedly showed some mixed myeloid-plasmacytoid antigenic expressions. The objective of this study was to verify and extrapolate this primary observation, as well as to discuss its possible significance.
Methods: There were studied marrow samples from 5 children with T-ALL during MRD assays on maintenance treatment. In all protocols there were included surface CD3, CD5, CD123, CD45 in combination with a testing antigen including CD1c (BDCA1), CD2, cytoplasmic CD3 (cCD3), CD4, CD7, CD8, CD11b, CD11c, CD16, CD19, CD20, CD33, CD56, TCRab, TCRgd and CD138. mDC were defined by BDCA1+, CD33+, CD11c+ and pDC by CD123++, CD11b- and CD4+. The propositus population under scrutiny was CD5+ and CD3- and will be referred as x. The percentages of CD5+ dendritic cells were assayed within this population and defined as CD5+mDC/x and CD5+pDC/x. Total mDC and pDC were measured within CD45+ cells. The expressions of testing antigens were estimated within CD5+mDC and total mDC as well as within CD5+pDC and total pDC cells comparatively to assay any differences. Median values of 5 tests are reported. Expressions below 5% were considered negative (n).
Results: x/WBC=0.23%, CD5+mDC/x=65.2%, CD5+pDC/x=31.8%, mDC/WBC=0.7%, CD5+mDC/WBC=0.14%, pDC/WBC=1.07%, CD5+pDC/WBC=0.08%, CD5+/mDC=29.7%, CD5+/pDC=10.2%, CD33+/CD5+pDC=92.3%, CD33+/pDC=21.8%, CD11c+/CD5+pDC=53.2%, CD11c+/pDC=n, CD4+/CD5+mDC=48.2%, CD4+/mDC=n, CD7+/CD5+pDC=13%, CD7+/pDC=16.2%, CD2+/CD5+pDC=91.8%, CD2+/pDC=36.2%, cCD3/CD5+mDC and CD5+pDC=n, CD56+/CD5+pDC=14.5%, CD56+/pDC=n. The rest of the tested antigens were negative.
Conclusions: The propositus population was below 1% and imitated T-ALL MRD (CD5+, CD2+, CD4+, CD33+). However it was cytoplasmic CD3 negative and consisted of CD5 positive mDC and pDC. CD5+pDC shared phenotypic features of mDC, with high expressions of CD11c and CD33, while CD5+mDC were CD4+, resembling pDC. The implications of this observation are twofold. Firstly MRD detection strategies in T-ALL should always be based on cCD3 and not only on surface T cell antigens, even if there exist aberrant coexpressions of CD5, CD4 and CD2 with CD33. Secondly the expression of CD5 should be recognized in both mDC and pDC. The phenotypic plasticity noted in these CD5+ dendritic subpopulations, raises possible issues of reciprocal ontogenetical plasticity between mDC and pDC and should be further investigated.

21 (Selected Best Poster Abstract Presentation)

Technology Transfer Using Harmonemia Concept of 10-Color 15-Antibody Lymphoma Screening Panel Developed in Canada and Implemented in Sweden

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Introduction: Immunophenotyping of lymphoid cell subset panel (ILCSP) for detection of lymphoma is the most common request at clinical flow cytometry laboratories. We have developed a 10-color 15 antibody lymphocyte population screening tube in Toronto, Canada and transferred the technology to Lund, Sweden, using the Harmonemia concept (1).
Materials and Methods: For validation, 60 samples were compared with routine ILCSP (two tube 10-color) in Toronto, and 45 samples were compared with three tube 8-color ILCSP in Lund. After the new panel was implemented in Lund, 300 samples were analysed.
0.5-1x10^6 washed cells were incubated with the antibody cocktail for 15 minutes. For lysis, VersaLyse was applied in Toronto and PharmLyse in Lund. 30-50,000 events were acquired on the Navios Flow Cytometer as previously described (2). To harmonize instruments between Toronto and Lund, target channels were determined in Toronto using Flow-set Pro and compensation was set using VersaComp beads (1). The same target channels were established on two Navios in Lund. Using Kaluza software, analysis templates were developed (one sheet for B-cell and one for T- and NK-cell populations). The reports are created automatically by copying the contents of a pre-programmed Kaluza "Information" window to an Excel sheet where numbers are combined with text into standard reports.
Results: The new screening panel consists of: CD4/kappa FITC, CD8/lambda PE, CD3/CD14 ECD, CD38 PC5.5, CD20/CD56 PC7, CD10 APC, CD19 APC-A700, CD5 APC-A750, CD57/CD23 PB, CD45KO. Employing Toronto target channels, only minimal adjustments of compensation matrix were necessary to obtain in Lund the same patterns of antigen expression. The validation of the new panel in both laboratories gave consistent immunophenotyping diagnoses and similar frequencies of lymphoid subsets by comparison to previous panels. Since the panel previously applied in Lund did not include NK-cell markers, the new panel gave more diagnostic information. In routine practice, a final report could be made for >90% of the samples, i.e. only a few samples needed additional immunophenotyping such as hairy cell or extended T-cell panel. By implementing this panel, the costs of ILCSP in Lund have been cut by 30%.
Conclusion: A 10-color 15 antibody panel can be a quick and efficient way to screen for aberrant lymphoid populations, especially useful in samples with low cellularity. The Harmonemia concept allows easy transfer of technology between laboratories.
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Expression pattern of CD25 in B-cell Acute Lymphoblastic leukemia (B-ALL) and its value in the minimal residual disease (MRD) monitoring

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Introduction: CD25, interleukin-2 receptor-α-chain, is shown to be expressed in B-ALL and found to be associated with BCRABL-translocation. However, its value in B-ALL MRD monitoring is unknown. We studied the expression pattern of CD25 and its usage in B-ALL MRD monitoring.
Methods: We studied the CD25-expression (fluorochrome-PECF594, clone- M-A251, BD) in 206 cases of B-ALL diagnosed using 10-color MFC immunophenotyping with 5-tube-antibody-panel. CD25-expression was correlated with recurrent cytogenetic abnormalities. Of 206, the post-induction (day 29-35) MRD was performed in bone marrow samples from 105 cases. We evaluated CD25-expression and its post-induction modulation in 49 MRD-positive samples. MFC was performed using bulk-lysis and stain method on Navios flow-cytometer and data was analyzed using Kaluza-software. The study was conducted in Tata Memorial Centre Mumbai according to guideline of institutional review board.

Results: Of 206 cases, 118 were childhood-B-ALL (age, 1-14 years) and 88 were adult-B-ALL (age, 14-64 years), (Male:Female – 153:53). Cytogenetics data was available in 201 cases (childhood-116 & adult-85), of them BCRABL-translocation was detected in 20%, ETV6-RUNX1 in 9.5%, TCF3-PBX1 in 9% and MLL-translocation in 2% cases. Of 206 diagnostic samples, CD25 was expressed in 42/206-20.4% (Childhood-BALL, 6.8% & adult-BALL 38.6%) samples. CD25 showed strong expression in 27/206-13.1% and partial/weak expression in 15/206-7.3%. In BCRABL-positive cases (n=40) CD25 was expressed in 65% and was significantly associated with BCRABL-translocation (*p*-value <0.001 & *r*=0.6). MRD was available in 105 cases and was positive in 49/105-46.7% (childhood-BALL, 31/70-44.3% & adult-BALL 18/35-51.4%) cases with median of 0.16% (range 0.002 to 84%). In 49 MRD-positive samples CD25 was expressed in 28.6% (Childhood-BALL, 8/31-25.8% & adult-BALL 6/18-33.3%). Of 40 BCRABL-positive cases MRD was available in 17 cases in which 11 were MRD-positive (64.7%). CD25 showed high frequency of expression (Total 5/11-45.5%, childhood-BALL 1/2 -50% & adult-BALL 4/9-44.4%) in MRD samples of BCRABL-positive cases. On comparison of CD25-expression between diagnostic with MRD-positive samples, it was lost in 46.7% and gained in 17.6% cases.
Conclusion: In BALL cases CD25-expression is significantly associated BCRABL translocation. It is a useful marker in the MRD detection in adult B-ALL, especially in BCRABL-positive cases.

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5 years PNH clone screening results of Russian patients

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Introduction: PNH clone can be a cause of hemolysis and serious devastating disease as paroxysmal nocturne hemoglobinuria (PNH) that needs to evaluate it before symptoms appear or to prove diagnosis. Because of this clone is rare event it requires testing sufficient number of patients. In 2011 in Russia PNH clone screening became systematic over the country and results of 5 years work is presented here.
Methods: Patients were tested on PNH clone when showed one of followed features: cytopenia or thrombosis, hemolysis, anemia, aplastic anemia (AA) or myelodysplastic syndrome (MDS). Patient was positive on PNH clone with size more than 0,01% on granulocytes. Clone size was evaluated by ICCS guideline flow cytometry method.
Results: 5711 patients were tested. All patients were divided on groups by main clinical/laboratory symptoms. In AA patients 51,1% from 1674 were with PNH clone. In other groups results were followed: MDS 16,4% from 1026, PNH suspicion 6,3% from 748, Hemolytic Anemia (HA) 9,4% from 969, Pancytopenia 21,1% from 219, Anemia 8,4% from 431, Thrombocytopenia 10,4% from 67, ITP 7,8% from 102, Hemolysis 10,2% from 49, Nepgropathology 2,5% from 79, Thrombosis 6,3% from 222, lympho(mielo)proliferative 10,1% from 79 and Other diseases 4,3% from 46.
The proportion of patients with PNH clone size >1% from all PNH positive patients in groups are: in PNH suspicion group it is 88% from 285, in AA group 59% from 855, in MDS 46% from 168, in HA 48% from 91, in Pancytopenia 52% from 44, in Anemia 28% from 36, in Lympho(mielo)proliferative diseases 2 patients from 8, in Thrombocytopenia 2 patients from 7, in ITP 0 from 8, in Hemolysis 2 patients from 5, in Nephropathology 1 patients from 2 and in Others 0 from 2.
The most interesting is a PNH clone dynamic results when three groups of patients were followed up. In group without PNH clone (n=385) in 12 months clone size was 1,1% (clone appeared in 36 patients with AA, 4 with HA and 4 with MDS). 60 patients with minor clone has showed clone size increasing from 0,33% to 2,32% in group in 30 months. In patients group with PNH clone >1% (n=147) in 31 months clone size has increased from 46,96% to 54,06% size.
Conclusion: PNH clone has to be evaluated constantly in patients from high risk groups and has to be followed up further due to possible appearing and growing.

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Minimal residual disease in acute myeloblastic leukemia evaluated by 8 color flow cytometry before and after allogeneic stem cell transplantation

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Minimal Residual Disease (MRD) monitoring is an important parameter to determine risk of relapse in acute myeloid leukemia (AML). Allogeneic stem cell transplantation (alloSCT) is a potentially curative treatment for AML patients, but the main cause of failure is the relapse. Therefore, MRD is important to discriminate patients with potential poor outcome after alloSCT. Herein, the survival curves of 91 AML patients who underwent alloSCT were analyzed according to MRD levels assessed by 8 color multiparametric flow cytometry (MFC) and were compared with other factors which could influence the survival.
Patients transplanted from December 2012 to February 2016 were evaluated for overall survival (OS), relapse free survival (RFS) and relapse incidence (RI), before alloSCT and at days +100 and D+200. Patient's median age was 33 years (1-59), and the median of follow-up was 10 months (2 – 38). MRD levels were from 0,01% to 4,34%. One to three tubes of 8 color monoclonal antibodies were used, based on Euroflow approach, by using 4 backbones (CD 34 PerCP Cy5.5, CD117PEcy7, HLA-DRV450, CD45V500) in combination with other markers observed in the diagnosis. 1.000.000 events were acquired in a FACSCanto II (BD)™. Data analysis was performed by Infinicyt (Cytognos) ™ software. Statistical analysis was performed by SPSS software (IBM)® including the parameters: MRD level (>0,1% or <0,1%), age (≤18 or > 18 years-old), disease status before SCT (initial, intermediate, advanced), donor (related or non related), stem cell source (BM, PBSC, UCB), conditioning regimen (myeloablative and not) acute and chronic GVHD.
There was no statistical significance on OS (p = 0,98), RFS (p = 0,85) and RI (p = 0,336) of MRD evaluation before SCT (n= 50). MRD evaluation (level > 0,1% or < 0,1%) on D+100 (n=80) after SCT showed significance on the probabilities of OS (0 versus 27%)(p<0,001), RFS (14 versus 38%)(p<0,001) and RI (86% versus 32%)(p< 0,001) respectively. On D+200 (n=31), the probabilities of OS were 0 versus 46% (p = 0,012), of RFS were 0 versus 52% (p<0,001) and of RI, 100% versus 11% (p< 0,001), respectively for MRD greater or less than 0,1%. In the multivariate analysis, none of the other parameters analyzed showed impact in survivor.
Flow MRD assessment before SCT could not effectively identify all individuals that would relapse after SCT. At D+ 100 and D+200flow MRD was useful to predict relapse. However, even at these time points, flow MRD had low sensitivity for this purpose.

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CD317 expression as a good prognostic factor or not in Chronic lymphocytic leukemia ?

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Introduction: Chronic lymphocytic leukemia (CLL) is a common type of leukemia in adults. Its clinical course is extremely heterogeneous, with some patients surviving without the need for a specific treatment while some others need an urgent therapy. Disease stage cannot differentiate progressive disease from a more stable disease course .Thus, various parameters determining prognosis independently of the disease stage have been developed. Various prognostic factors have been defined for CLL including ; such as cytogenetic abnormalities,CD49d, CD38 and ZAP70 expression etc. CD317 can be used as a prognostic factor in some studies. A relevant correlation between CD317 and CD38 negativity has been indicated. Limitations in usage of CD38 as a prognostic factor can be removed by evaluating with CD317.
Methods: 42 patients were diagnosed with CLL. CD38 and ZAP-70 expression levels were measured with five color flowcytometry. We added CD317 and aimed to examine correlation between CD38 and ZAP70 in our lymphoproliferative disease panel.
Result: We have examined 42 new diagnostics CD317 expression in CLL fact in our Hematology Lab.. In %80 of facts. CD38 has been found negative and %52 of CD38 negative facts, there has been CD317 expression.
Conclusion: In all of studies before, CD317 overexpression have been monitored in CD38 negative facts. In our study, overexpression of CD317 has been monitored only in half of facts. Our CD38 negative and CD317 positive facts are still being tracked without treatment. At the end of this tracking, there will be more clear findings if there is good prognostic factor or not.

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CD16+ variant of Acute Monocytic Leukemia? A malignant counterpart of CD16+ monocytes?

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Introduction: Acute Monocytic Leukemias (AML-M5) are diagnosed by a percentage over 20%, of monoblasts and promonocytes (mp%) by smear morphology. The immunophenotype conducted by flow cytometry, includes antigens with increased monocytic specificity, which are also expressed on normal monocytes. Monocyte classification has been based on the expression of CD16, discriminating CD16+ monocytes from classical CD14+CD16- ones. The aim of this study was to assess the expression of CD16 as a putative classifier of malignant monocytes in AML-M5 and correlate it with other classification and diagnostic factors.
Methods: There were reviewed data from 32 cases of AML-M5 including mp%, the percentage of blasts by flow cytometry (fc%) with CD45 and side scatter gating (blast gate), the expression of 35 antigens within blast gate (myeloperoxidase (MPO), lysozyme, cytCD68, cytCD66c, cytCD3, cytCD79a, cytCD13, Tdt, CD34, CD56, CD64, HLADR, CD14, CD16, CD33, CD15, CD7, CD13, CD2, CD19, CD10, CD20, CD4, CD117, CD36, NG2, CD38, CD123, CD11c, CD11b, CD9, CD25, CD71, CD41) karyotype, FISH and molecular analysis conducted by routine diagnostic methods. The intensity of CD45 of monocyte blast gate was assessed by the normalized ratio of CD45 MFI to lymphocyte value, set to 1. Specifically the expression of CD16 was used as a classifier of AML-M5, with a cut-off value of 15%, discriminated CD16+M5 and CD16-M5 AML in two groups, which were compared by morphology, flow cytometry, cytogenetics and NPM1 mutation PCR status. Values of mp% and fc% were also compared. Blood samples from 6 blood donors were assessed for the expressions of tested antigens between classical and CD16+ monocytes and compared with the expressions on leukemic monocytes.
Results: CD16+M5 (n=7/32, 22%) and CD16-M5(n=25). There were observed morphological differences in CD16+M5, with increased phagocytosis, excessive cytoplasmic vacuolization (Burkitt-like) and increased cytoplasmic protrusions. The phenotypic differences that reached statistical significance in CD16+M5 were increased CD45 intensity, increased CD14% and negative/low MPO. In CD16-M5 there was 50% discordance between fc% and mp%, while there was no discordance in CD16+M5. No other antigens differed significantly between the two groups. There were not significant karyotype and NPM1 mutation rate differences. Normal donor CD14+CD16+ monocytes had negative/low MPO expression compared to MPO+ classical monocytes.
Discussion: CD16+M5 consisted 22% of AML-M5, with characteristic CD45bright CD14 bright"mature type" blast gate, Burkitt-like morphology and negative MPO. They could form a provisional CD16+ AML-M5 variant. Intermediate CD14+CD16+ MPO- monocyte could be a putative normal counterpart cell.

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Multiparametric flowcytometry for immunofenotyping of rare mast cells in bone marrow

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Introduction: Systemic mastocytosis is a rare mast cell disease due to clonal, neoplastic proliferation of mast cells (diagnostic criteria World Health Organization (WHO) classification). One out of four minor criteria is the expression of CD2 and/or CD25 on mast cells. The low mast cell frequency in both normal bone marrow and bone marrow from patients with mastocytosis demands for a reliable and sensitive immunophenotypic assay. We performed a retrospective review of our flow cytometric mast cell analysis on bone marrow from patients with and without the diagnosis of systemic mastocytosis to evaluate the performance of the flow cytometric test in our laboratory.
Methods: A multiparametric flowcytometric analysis was performed on bone marrow from both patients with confirmed systemic mastocytosis (n = 22) and a control population (n=81). The markers CD33, CD34, CD45, CD117, CD203 were used to separate and quantitate the total mast cell population while CD2, CD25, CD30 were used to discriminate between normal and aberrant mast cells. Flow cytometric results were expressed as a ratio of median fluorescence intensities (MFI) obtained by dividing MFI CD2, CD25 or CD30 on mast cells by the MFI of CD2, CD25 or CD30 respectively on an internal control population (lymphocytes). A marker was scored positive (MFI ratio >10) or negative (MFI ratio <10). Sensitivity and specificity were calculated.

Results: In patients with systemic mastocytosis a mast cell frequency of 0.014-2.4% was observed, in the control population a frequency of 0.0036-0.68% was found. Due to low mast cell frequency in the bone marrow more than 50 % of tests that were performed could not be included and therefore the standard amount of analyzed white blood cell was increased in our daily routine. The specificity of all three tested aberrant markers was very good (≥96%) with CD25 having the greatest sensitivity (94%), for CD2 and CD30 sensitivity was respectively 72% and 61% meaning that CD2 and CD30 were frequently scored negative on mast cells in the patient population. Sensitivity of CD30 could be increased to 83% by setting the MFI ratio cut-off value for positive scoring to 7.5.
Conclusions: Multiparametric flow cytometry is a reliable method to quantify rare mast cell events in bone marrow. MFI ratio is an objective analysis method for scoring aberrant markers as positive/negative. CD30 is, beside CD2 and CD25, also a specific and sensitive marker for neoplastic mast cells and helpfull in the diagnosis of systemic mastocytosis.

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Phenotypic analysis of HSC, B and plasma cells in transplanted multiple myeloma patients
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Introduction: Multiple myeloma (MM) is characterized by accumulation of clonal plasma cells (PCs) in bone marrow (BM). The common treatment approach in younger (≤ 65 years) MM patients is autologous transplantation (ASCT) of peripheral blood haematopoietic stem cells. Detailed assessment of cell populations that may be source of myeloma cell may help to find possible connections with the clinical state of the patients and eventually predict response to the treatment.
Aim: Phenotypic analysis of haematopoietic stem cell (HSC), B cell and PC compartments in different time points of the treatment in multiple myeloma patients.
Patients and methods: Total of 17 MM patients were analysed by polychromatic flow cytometry in time of diagnosis, separation PBSCs, after 1st month (M+1) and after 3rd month (M+3) after ASCT. Analysis of $CD34^{+}CD19^{-}$ HSCs, subpopulations of $CD19^{+}$ B cells (proB, preB1 preB2, immature and mature forms), plasmablasts (PBs) and PCs was done in peripheral blood (PB), BM and apheresis product.
Results: Relative count of HSCs was the lowest at the time of diagnosis [median 0.37 % (range 0.08-1.11)], the highest in the apheresis product [1.65 (0.13-6.24)], and still higher in M+3 [0.62 (0.03-0.87)] than in time of diagnosis. Total number of B cells was 2.31 % [(0.45-6.65)] in time of diagnosis, the lowest in apheresis product [0.14 % (0.02-0.42)] and the highest in M+3 [(5.41 % (0.04-10.73)], where mostly preB1 subtype dominate the samples. There was found increasing number of immature B cells from new diagnosis to M+3 [(3.02 vs. 14.01)]. As expected, the highest number of pathological PC [5.5 % (3.65-41.72)] was found in BM at the time of diagnosis. Pathological $CD19^{-}$ PCs were detectable only in 11.8 % (2/17) of patients in apheresis product. PCs were detected in all analysed samples in M+3 [0.36 % (0.04-2.16)] and in 63.6 % (7/11) of patients clonal PCs were detected with median 10 % [(0.2-86.0)]. Although 54.5 % (6/11) of patients reached the complete remission (CR), the immunophenotype CR (iCR) was confirmed only in 36.4 % (4/11) of them. Circulating PCs were identified in 65 % (11/17) of patients [0.01 % (0.0 -1.93)] in time of diagnosis, but were not detected in any patients in M+3.
Conclusion: Treatment of MM patients decreased marrow myelosuppression by elimination of clonal PCs. That resulted in increased number of HSCs and less mature B cell forms. Reaching of iCR may predicts survival of MM patients.

29 (Selected Best Poster Abstract Presentation)

The CD20+ CD28- CD117- phenotype of myeloma plasmacells is associated with t(11;14)
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Introduction: The diagnostic and prognostic value of surface antigen expression by Multiple Myeloma (MM) PlasmaCells (PC) at disease onset is still not entirely elucidated. Namely, the role of CD20 expression on MM PC is debated. Molecular cytogenetic analysis performed on highly purified PC is able to disclose translocations associated with standard risk (i.e. t(11;14) and t(6;14)) or high risk disease (i.e. t(4;14) and t(14;16)). We have retrospectively analyzed a recent series of MM patients at disease onset to look for possible associations between PC immunophenotype, focused on CD20 expression, and cytogenetic findings.

Methods: PC immunophenotyping was performed on dissociated bone marrow aspirate particles from 124 MM patients at onset with a standardized 8-color 2-tube protocol including CD20 H450/ CD45 H500/ cyKappa FITC/ cyLambda PE/ CD138 PerCP-Cy5.5/ CD56 PE-Cy7/ CD38 APC/ CD19 APC-H7 and CD28 H450/ CD45 H500/ CD81 FITC/ CD117 PE/ CD138 PerCP-Cy5.5/ CD27 PE-Cy7/ CD38 APC/ CD19 APC-H7 with Fix-and-Perm treatment and Infinicyt analysis. Highly purified PC from the same samples were obtained by autoMACS Pro Separator (Miltenyi) and processed for FISH analysis with a panel of at least 5 MM-associated translocations.
Results: Twenty-seven patients out of 124 showed some IgH rearrangements at FISH analysis (17 with t(11;14), 1 with t(6;14), 3 with t(4;16) and 6 with t(4;14)). Expression of CD20 was demonstrated in 21 cases. Stratification of CD20 expression along with negativity for CD28 and CD117 showed that 8 cases out of 21 had t(11;14), whereas 9 cases had CD20+ but with variable CD28 and CD117 expression and were negative for t(11;14) (Fisher's exact test: p=0.0089). CD20 expression was consistently negative in all the other MM-associated translocations.
Conclusion: A MM PC CD20+ CD28- CD117- phenotype is significantly associated with t(11;14) and may be useful for a preliminary prediction of this standard risk cytogenetic abnormality.

30 (Selected Best Poster Abstract Presentation)

Multicentric evaluation of the current medical indication for Paroxysmal Noctural Hemoglobinuria diagnostic screening

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Introduction: Efficiency of the consensus guidelines for the diagnostic screening of paroxysmal nocturnal hemoglobinuria (PNH) by flow cytometry (FCM) has not been investigated, althoug have been proposed in 2010. Accordingly we have evaluated the efficiency of the current consensus medical indications for diagnostic screening of PNH by FCM
Methods: Overall, information about 3,938 samples from an identical number of individuals prospectively submitted between January 2011 and December 2014 for diagnostic screening of PNH by flow cytometry was collected at 24 flow cytometry laboratories in Spain which participate in the PNH-External Quality Assurance Program of the Iberian Society of Cytometry (1,718 samples) plus one reference laboratory in Sao Paulo, Brazil (2,220 samples). The following GPI-associated markers were analyzed: FLAER (analyzed in 87% of the cases), CD14 (98%), CD16 (37%), CD24 (93%), and/or CD157 (5%). In those cases with GPI-deficient mature neutrophils and monocytes, expression of CD59 (100% of cases) was also analyzed on red blood cells.

Results: Overall, diagnostic screening based on consensus medical indications was highly efficient (567 PNH^+/3,938 screened cases; 14% PNH^+ samples) both in the multicenter setting in Spain (10%) and reference laboratory in Brazil (16%). The efficiency was higher among samples screened using FLAER (11% vs. 7%; $p<0.01$).
Estimated annual incidence of new PNH cases was of ≈2.5 cases/million individuals per year. Among these, frequency of PNH^+ cases was higher among patients screened because of bone marrow (BM) failure syndrome (33%), particularly among those with mainly aplastic anemia (243/541; 45%) and to a less extent also myelodysplastic syndrome (26/266; 10%). When excluding BM failure syndromes, the annual incidence of PNH was of 0.6 cases/million individuals/year. Among those individuals without previous diagnostic of BM failure, the most efficient medical indications for PNH screening included: hemoglobinuria (35/73; 48%), unexplained cytopenias including anemia (88/393; 22%), non-immune hemolytic anemia (71/382; 19%), and thrombosis associated to (non-hemolytic) anemia and/or another cytopenia (10/73; 14%). PNH^+ cases was less commonly observed among unexplained cytopenias in the absence of anemia (39/772; 5.1%) chronic myeloproliferative neoplasms (1/21; 4.8%), or unspecified anemia (17/468; 3.6%). In contrast, only a minor fraction of the patients who had been submitted for PNH testing because of unexplained thrombosis in the absence of cytopenia were positive (3/800; 0.4%).
Conclusions: Our results demonstrate that the current medical indications for PNH screening by FCM are highly efficient, although improved screening algorithms are needed for patients presenting with thrombosis and normal blood cell counts.

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Extensive immunopnenotyping in a case of 'mature' Blastic Plasmacytoid Dendritic Cell Neoplasm

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Introduction: Blastic plasmacytoid dendritic cell neoplasm (BPDCN) is a very rare aggressive hematologic malignancy, characterized by massive bone marrow (BM) and extramedullary localization, with typical dermal and lymph node tropism. On the basis of clinical and immunophenotype features, BPDCNs have been recently classified in “immature”, “intermediate”, and “mature” forms.
Methods: Eight-coulor flow cytometry analysis was performed in a case of BPDCN with $CD4^+$ $CD56^+$ $CD123^+$ $CD34^-$ $CD117^-$ “mature” phenotype. The patient displayed a “mature” clinical behaviour too, initially presenting with wide involvement of skin, lymph nodes, and rhynofaringeal tissues, then rapidly developing overt leukemia. Complete remission was obtained with acute lymphoid leukemia-like therapy.
Results: BPDCN mmunophenotype was studied extensively on both peripheral blood and BM samples, and showed middle-sized blasts $CD45^{dim}$, $CD4^+$, $CD10^+$ $CD38^+$ $CD45RA^+$ $CD56^+$ $CD123^+$ $HLADR^+$ $CD1a^-$ $CD3^-$ $CD5^-$ $CD8^-$ $CD11b^-$ $CD11c^-$ $CD13^-$ $CD14^-$ $CD15^-$ $CD16^-$ $CD19^-$ $CD20^-$ $CD22^-$ $CD25^-$ $CD33^-$ $CD34^-$ $CD36^-$ $CD64^-$ $CD66c^-$ $CD117^-$ $CD235a^-$ $cytCD3^-$ $cytCD22^-$ $cytCD41^-$ cytCD61- $cytCD79a^-$ $cytMPO^-$, partly $CD2^+$ $CD7^+$ TdT^+. Immunohistochemistry on lymph node and BM biopses showed the same data, and further detected BCL2 overexpression, in the absence of BCL2 gene rearrangement or amplification, as detected by FISH. A cluster of $CD45^{dim}$ $CD4^+$ $CD10^+$ $CD56^+$ $CD123^+$ cells was also detected in the cerebrospinal fluid, coherently with occult central nervous system localization.
Conclusions: The finding of CD2, CD7, CD10 and TdT positivity, BCL2 overexpression, as well as the clinical response to lymphoid-oriented treatment, seem to confirm the hypothesis that neoplastic cells from BPDCN can originate from a multipotent lymphoid-oriented precursor, with a possibly preferential differentiation into “mature” subtype.

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Early T-cell Precursor acute leukemia(ETP) with immunophenotypic transformation to myelogenous leukemia with minimal differentiation,during induction therapy

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Introduction: A case of Early T-cell precursor leukemia (ETP) with negative myeloperoxidase (MPO) and positive myeloid antigens is described, with a dramatic phenotype change during induction therapy with disappearance of cytoplasmic CD3 and the appearance of cytochemical myeloperoxidase.
Methods-Results: A 10 years old boy, initially hospitalized for dental abscess, showed leukopenia, neutropenia and anemia. The bone marrow was infiltrated with 83% blasts with intermediate CD45, cytoplasmic CD3+, CD1a-, CD2+, CD4-, CD8-, CD5-, CD7+, CD13+, CD34+, CD117+. During this phase the cytometric as well as the cytochemical MPO were completely negative. The above characteristics were compatible with the recently recognized form of T-cell acute lymphoblastic leukemia (T-ALL), Early T-cell Precursor leukemia(ETP), a T-ALL subgroup with a poor prognosis. The karyotype showed a chromosome 5 rearrangement with prevalence of q13 and q14q35 deletions. Interphase molecular cytogenetics (FISH) was negative for BCR/ABL fusion and MLL rearrangement. The molecular analysis for FLT3 gene mutations was negative.The patient was treated by the ALLIC BFM2009 protocol. At day 15, a bone marrow specimen showed 73,4% blasts which were positive for CD2 CD15, CD33, lysozyme, CD13 and CD7dim. Cytoplasmic CD3 and the immature markers CD34 and CD117 which were highly expressed at diagnosis, all became negative. During this phase though the flow cytometric MPO was negative, the cytochemical MPO was found positive in 15% of blasts. According to WHO 2008 classification, the blast phenotype was compatible with myelogenous leukemia with minimal differentiation. At treatment day 33 blasts were 16.7%, retaining CD2 strong positivity, while CD7 became completely negative. Cytoplasmic CD3 and flow cytometric MPO remained negative while positive cytochemical MPO was observed in 8% of blasts.The karyotype was normal, showing cytogenetic remission without any malignant clone.There was not any modification in the treatment protocol, while 15 days later the blasts were 0.9%.
Conclusions: The points of interest in this case are: 1) the loss of cytoplasmic CD3, which constitutes a key element in T-ALL diagnosis. Immature antigens like CD34, CD99 and TdT, have been known to be affected by treatment in T-ALL, however the loss of cytoplasmic CD3 has not been thoroughly studied especially in ETP 2) the discordance between cytometric and cytochemical MPO leaves open questions about the standardization of this important diagnostic element, especially in an era where flow cytometry may be the only method used in diagnosis 3)the response to a T-ALL treatment protocol despite the myeloid phenotypic change.

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Flow cytometric evaluation of paroxysmal nocturnal hemoglobinuria clone sizes in patients at risk.
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Introduction: Paroxysmal nocturnal hemoglobinuria (PNH), a rare clonal hematopoietic stem cell disorder, is characterized by a somatic mutation in the PIGA gene, leading to a deficiency of proteins linked to the cell membrane via glycophosphatidylinositol (GPI) anchors. The lack of GPI-linked proteins results in chronic, uncontrolled complement activation leading to systemic complications, mainly through intravascular hemolysis and platelet activation. Thromboembolism is the most common cause of mortality in patients with PNH. Therefore it is important to identify patients with PNH early within the following PNH high-risk groups: Coombs-negative hemolytic anemia; hemoglobinuria; unexplained thrombosis; unexplained cytopenia; aplastic anemia (AA) and myelodysplastic syndrome (MDS). Flow cytometry is the diagnostic method of choice for reliable PNH testing.
The objective of this retrospective study was to investigate in a larger cohort of patients the clinical presentations that alert physicians to request flow cytometric PNH testing and to evaluate how the high-risk groups are represented by the occurrence of PNH clones.
Methods: A total of 158 patients had blood samples collected for flow cytometric PNH testing between January 2015 and April 2016. Those were retrospectively reviewed for reasons of testing, lactate dehydrogenase (LDH) levels, age distribution, and size of the GPI-deficient clone.
Results: PNH testing was particularly performed in patients with unexplained thrombosis (25%), AA (23%) and unexplained cytopenia (15%). PNH testing was less frequently requested for patients with Coombs-negative hemolysis (12%), MDS (7%), or hemoglobinuria (1%). Patients tested for PNH were generally between 35 and 74 years of age (64% of patients). Granulocyte/monocyte PNH clones ≥ 0.03% were detected in 27 of all 158 tested patients (17%): 18 of 36 AA patients (50%), 4 of 23 unexplained cytopenia (17%), 1 of 19 Coombs-neg. hemolysis (5%), 1 of 40 unexplained thrombosis (3%) and 3 of 27 in other or unknown patients (11%). PNH clone size in AA and unexplained cytopenia patients was mainly <10% (67% and 100%, respectively). PNH clones over 10% were detected in 8 patients. In 25 of 27 patients PNH clones were also detected in the erythrocytes, but those were smaller in clone size when compared to the granulocyte/monocyte clone size. PNH clones occurred in patients with normal as well as high LDH-level ranges.
Conclusions: The data of the present study show that treating physicians request flow cytometric PNH testing in all populations at risk for PNH and confirm the presence of PNH clones in these populations.

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Clinical and Immunophenotypic features of Acute Myeloid Leukaemia (AML) with NPM1 and FLT3 gene mutations: A retrospective analysis

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Introduction: NPM1 and FLT3 gene mutations are crucial prognostic indicators in patients with AML with recent reports indicating their presence can be predicted using immunophenotypic profiles. We studied 41 AML patients with NPM1 and FLT3 mutations to ascertain their immunophenotypic profiles along with the karyotypes.
Methods: Immunophenotyping profiles obtained from 41 AML patients diagnosed at the Haemato-Oncology Diagnostic service in Sheffield between 2012 – 2016 and identified as having NPM1 and FLT3 mutations were retrospectively analysed. Patients were categorized into three groups: 13/41(32%) NPM1+ FLT3 ITD-; 12/41 (29%) NPM1- FLT3 ITD+ and 16/41 (39%) NPM1+ FLT3 ITD+. These groups were then further sub-categorized on the basis of karyotype (normal karyotype and abnormal karyotype).
Results: Of the 13 NPM1+ FLT3 ITD- cases, only 5 (38%) showed HLA-DR expression with none (0%) having CD34 expression, 3/13 (23%) had an abnormal karyotype. Whereas, of the 12 NPM1-FLT3 ITD+ cases 12/12 (100%) were positive for HLADR and CD34 with 7/12 (58%) having an abnormal karyotype. In the NPM1+ FLT3 ITD+ group (n=16), 9/16 (56%) were positive for HLA-DR, 3/16 (19%) CD34+ and 1/16 had an abnormal karyotype.
This study also found that, irrespective of NPM1 mutation status, 17/28 (61%) FLT3 ITD+ patients expressed CD7 compared to 2/13 (15%) FLT3 ITD- patients.
NPM1+ FLT3 ITD- patients also had a higher median age disease onset (median age 60yrs; 48yrs and 52yrs for NPM1- FLT3 ITD+ and NPM1+ FLT3 ITD+ respectively). NPM1- FLT3 ITD+ patients had significantly higher WBC counts (124 x 10^9/L compared to <66 x 10^9/L for the other two groups) and a higher incidence of an abnormal karyotypes (7/12 compared to only 4 in the NPM1+ FLT3 ITD- and NPM1+ FLT3 ITD+ groups combined). No Auer rods were found in NPM1+FLT3 ITD- (0/13) whereas Auer Rods could be found in the NPM1- FLT3 ITD+ and NPM1+ FLT3 ITD+ groups (5/12 and 4/16 respectively).
Conclusions: This study has shown that NPM1-FLT3 ITD+ AML cases are CD34+ and HLADR+ , have a higher white cell count and more likely to be karyotypically abnormal than if the case was NPM1+ with or without a FLT3 ITD mutation. Furthermore, AML cases with a FLT3 ITD mutation (irrespective of NPM1 mutation status) are also more likely to be CD7+ than FLT3 ITD- cases. Finally, our data suggests that Auer rods are not seen in NPM1+FLT3 ITD- cases.

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Aneuploidy incidence and old and new plasmacell phenotype markers analyzed with multiparametric flow cytometry in Multiple Myeloma and MGUS

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Introduction: we used multiparameter flow cytometry to look for old and new plasmacellullar (PCs) markers, such as extrusion pumps of drugs (CD243) and cito-chemokine receptors, (CD197, CD191, CD192, CD196, CD183, CD184, CD126) involved in cellular interactions and adhesion. We studied the different expression between normal and neoplastic PCs in multiple myeloma, and the different expression between multiple myeloma and MGUS; the incidence and prognostic significance of the analysis of the cell cycle and aneuploidy, in order to support the actual efficacy of therapies.
Method: 35 subjects suffering from Multiple Myeloma and MGUS: CD56+, CD117+, CD200+, CD20+, CD33+, CD27+ and CD28+.
DRAQ5 dye to identify nucleated cells, throught absolute counting beads.
Concentration gradient through Lymphoprep™ .
Selection of the plasmacell population (CD138+) through MACS SORTING (Miltenyi Biotec).
Phenotypic analysis by multiparametric flow cytometry Navios™ Beckman Coulter (Ca,USA) and aneuploidy incidence by Cycloscope™ kit (Cytognos, Salamanca, Spain).
Statistical analysis using MedCalc software.
Results: The PCs of Multiple Myeloma and MGUS show a similar expression of each antigen, with an increase of CD197(P≤0,2), CD184 (P ≤ 0,07) and CD126 (P ≤ 0,08) in cases of MGUS, while CD192 (P ≤ 0,0002) and CD 243 (P ≤ 0,008) in cases of MM. MFI Statistical significance (P ≤ 0,05) .Comparing immunophenotypic expression of the examined molecules between normal and neoplastic PCs of MM, higher percentage and Mean Fluorescence Intensity values were detected on tumor PCs, for each molecules, in relation to their expression on the normal ones.On 17 bone marrow samples, suffering from Multiple Myeloma and MGUS, through the analysis of Index

DNA and the performance of the cell cycle, we obtained a total presence of Pseudodiploid patients.
Conclusion: Analysing the immunophenotypic expression of receptor molecules and extrusion pumps, we found an increased expression of these molecules on neoplastic PCs of Mieloma, compared to the normal ones. On the other hand, no significant difference was found, comparing the expression of the same samples of Multiple Myeloma and MGUS.Cell cycle and aneuploidy analysis of all samples showed a condition of pseudodiploidy with a more difficult prognosis, even considering the advanced age of the subjects.

36 (Selected Best Poster Abstract Presentation)

The use of DRAQ7 in PNH clone detection
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Introduction: PNH is a rare hematopoietic stem cell disorder characterized by a somatic mutation in the PIG-A gene resulting in deficiency of the GPI-anchor that links proteins to the membrane. At present, analysis of the GPI-anchored antigens by flow cytometry is the gold standard for detection of PNH clones in diagnostics and monitoring of PNH patients. The rapid and sensitive multi-parameter detection renders flow cytometry to a powerful tool to detect not only large but also small GPI-impaired (type II cells) and negative (type III cells) clones. However, pre-analytical procedures can strongly influence clone detection by dead and apoptotic cells causing non-specific binding and aggregation. To avoid under- or overestimation of clone size a viability dye should be used to exclude these cells. DRAQ7 is a fluorescing viability dye that does not enter intact cells. But, when the membrane integrity is compromised, it enters and binds readily to nuclear DNA to report cell death, reducing the potential for false-positives.
Methods: PNH clones in monocytes and granulocytes in blood of PNH patients were determined by using our PNH screening panel (Flear-Alexa488, CD55-PE, CD14-ECD, CD33-PECy7, CD59-APC, DRAQ7, CD24-APCA750, CD15-PB, CD45-BV510). Erythrocytes were eliminated prior to or immediately after MoAb-incubation in a standard procedure using Pharmlyse. The influence of apoptotic or dead cells on total PNH clone size as well as in the type II and type III PNH cells was evaluated.
Results: Influence of dead cells on monocytes and granulocytes was determined in 45 blood samples by using the PNH screening panel containing DRAQ7. In contrast to erythrocyte lysis by stain-lyse-wash procedure, lysis prior to staining results in high cell death. By elimination of the dead cells (DRAQ7-positive), monocytes decreased in percentages and number. The percentages of neutrophils were not changed, but the number decreased too. The percentages of total PNH clone size within monocytes and neutrophils were not affected, but the number of total PNH cells decreased, that was the largest in monocytes. However, the percentages of type II and type III PNH neutrophils were affected.
Conclusion: We showed that DRAQ7 is a suitable viability dye to determine the dead and apoptotic cells in PNH patients. Monocytes appear to be more sensitive for the pre-analytical setup than granulocytes. In monocytes the influence of dead cells on PHN clone size is evident. This effect appeared to be most pronounced in samples with small PNH clones of less than 5 %.

37 (Selected Best Poster Abstract Presentation)

Practical application of a flow cytometric scoring system for myelodysplastic syndromes in a routine diagnostic setting
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Introduction: Della Porta and colleagues proposed in 2012 a simple flow cytometric scoring system (FCSS) to assist in the diagnostics of myelodysplastic syndrome (MDS). Points are given for four different parameters involving the myeloblast-related and B-progenitor-related clusters, and the granulocytes.
The aim of the present study was to test the practical application of the FCSS in a routine diagnostic setting.
Methods: In a retrospective study we analyzed all bone marrow samples sent for flowcytometric analysis with a suggestive diagnosis of MDS or acute leukemia within a year (n=81). FCSS was calculated for all eligible samples and evaluated in relation to the final diagnosis of the patient (n=35, > 1.5 year follow-up). Exclusion criteria were acute myeloid leukemia (AML) at sampling time or less than 400 collected CD34+ events.
Results: The study suffered a high degree of sample exclusion. Correct estimation of the lymphoblast population in a 5 % level requires > 400 CD34+ events. With a routine limit of 30.000 total leukocytes, this criterion was frequently reason for exclusion. We suggest using a stopping criterion during data collection on 1000 CD34+ events to ensure valid data for calculation of the FCSS.

In 7 cases the pathologist had not been able to reach a diagnosis from the first marrow sample. 5 was subsequently diagnosed with MDS (corresponding to 29 % of the MDS cases). The 2 remaining was discharged with persistent cytopenias but unlikely mds. All 7 samples were classified correctly by FCSS.
All of the 3 samples with false positive analysis results belonged to the group of chronic myeloid cancers. Only one sample from this group was correctly scored as non-mds in our study. A case of ET only obtained a single point for high lymphocyte/myeloblast CD45 ratio. The false positive included: One PV case with a small atypical myeloblast population with relatively low SSC and high CD45 and no lymphoblasts; one myelofibrosis with later transformation into AML; and one CMML1 with low number of lymphoblasts and a high lymphocyte/myeloblast CD45 ratio.
Conclusions: The FCSS seems to have poor ability to discriminate myelodysplastic syndrome from other myeloid cancers. On the other hand, it performed very well in determining bone marrow changes deriving from non-neoplastic conditions or lymphomas. Furthermore, the implementation of the FCSS is simple, objective, and only requires a change in the number of collected events from our current analysis setup.

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Correlation between the Embryonic Stem Cells Antigens (ESCA) expression and Lin 28/let 7 feedback loops in acute myeloid leukemia (AML)

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Introduction: AML is a clonal disorder of hematopoietic stem and progenitor cells (HSPCs) with stepwise acquisition of somatic mutations that cause abnormal cell growth and differentiation. The disease initiation and progression is due to a subpopulation called leukemia stem cells (LSCs) that show an extensive proliferative potential and limited differentiation capacity.
The aberrant transcriptional circuits drive oncogenic expression programs that affect the switch between self-renewal and differentiation. Among ESCA there are multiple stem cell-specific transcription factors, i.e. OCT4, SOX2 and Nanog, which are involved in maintaining of LSC self-renewal capacity and pluripotency. The activity of these transcription factors is tightly regulated by the miRNAs. Recent data indicate the existence of a direct relationship between the pluripotency factor LIN28 and let-7 miRNAs; factors well-known for controlling self-renewal and differentiation processes. In addition, recent evidences show that Lin28 is an oncogene that drives tumorigenesis in part by suppressing let-7 (L. H. Nguyen, H. Zhu, 2015). The aim of our study is to investigate the role of ESCA in regulation of HSPC fate through the Lin28/let-7 Axis.
Methods: Multicolor flow cytometric analyses were performed to evaluate expression of three ESCA on HSPCs and LSCs from 20 healthy donors and 50 AML. Correlations with the relative expression of let7 and Lin28 have been performed in part of these cases (2 healthy donors and 14 AML patients). The quantification of let7 and Lin28 was performed by RT-PCR. Informed consent was obtained from donors and patients prior to the study. Technical validation has been done initially in leukemic cells lines.
Results: The preliminary experiments performed on cell line HL60 shown an increase level of expression of let-7 miRNA during the cell differentiation process assessed by upregulation of CD11b antigen expression after retinoic acid exposure . Simultaneously, expression of the ESCA expression was down-regulated. These results proves that a negative feedback loop exist between let-7 and Lin28, and shows a positive correlation between Lin28 and ESCA expression. In AML samples, we found a decreased level of expression for all members of let-7 miRNA family, compared with the levels detected on the normal samples, which was associated to an increased expression of Lin28 and of the ESCA assessed.
Conclusions: These data support hypothesis that Lin28 operate as an oncogene that drives leukemogenesis in part by suppressing let-7 and, consequently by blocking differentiation. ESCA expression in AML blasts can be used as surrogate makers for LSC detection and follow-up.

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Absence of CD66c expression excludes the possibility of BCR-ABL1 translocation in Bcell precursor acute lymphoblastic leukemia (BCPALL)

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Introduction: CD66c (CEACM6) is a myeloid-lineage associated antigen expressed aberrantly by blasts in B-cell precursor acute lymphoblastic leukemia (BCPALL). A few studies have claimed that CD66c expression is strongly associated with BCR-ABL1 translocation in BCPALL and more recently, also shown to be associated with CRLF2-positive BCPALL. However, these studies are done in a small cohort of patients and need further validation in large number of patients. Therefore,we studied the expression pattern of CD66c in a large cohort of 474 BCPALL cases and determined its association with BCRABL1 translocation in our patients.
Methods: Expression pattern of CD66c (Fluorochrome: PE, Clone: KOR-SA 3544, Beckman Coulter) was studied in the diagnostic samples from 474 BCPALL cases (age 1-67 years & M:F, 2.02). The diagnosis was made as per WHO-2008 classification and flow cytometric immunophenotyping (FCI) was performed using 9-color comprehensive antibody panel on Navios cytometer (Beckman Coulter, BC) and data was analyzed using Kaluza-v1.3 (BC). Recurrent translocations were studied by FISH and BCR-ABL1 translocation was studied using Abbott Mol LSI dual-fusion probes.
Results: CD66c was positive in 56.3% diagnostic samples of BCPALL, followed by CD15 in 14.5%, CD13 in 11.9%, CD33 in 5.6% & CD117 in 1.3% samples. In 474 samples, its expression pattern was strong, intermediate, partial, weak and heterogeneous in 20.3%, 1.7%, 8.6%, 2.3% and 23.4% samples respectively. On cytogenetics study, BCR-ABL1 translocation was positive in 59 (12.4%) cases. The sensitivity, specificity, positive predictive value and negative predictive value of CD66c expression in predicting BCR-ABL1 translocation was found to be 19.7% (95% CI- 14.88% to 25.22%), 95.2% (95% CI- 91.60% to 97.59%), 81.4% (95% CI- 69.09% to 90.31%) and 52.8% (95% CI- 47.84% to 57.66%) respectively. On correlation study, spearman correlation was r=0.19 with p-value<0.001 suggesting weak but statistically significant positive correlation between CD66c expression and BCRABL translocation.
Conclusion: CD66c is the most common myeloid antigen expressed aberrantly in BCPALL. CD66c expression has low sensitivity but very high specificity with high positive-predictive-value for BCR-ABL1 translocation in BCPALL. Thus, CD66c expression can be reliably used to exclude the possibility of BCR-ABL1 translocation in resource limited settings and in cases where cytogenetics study is not available.

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Thrombotic auto-immune disorders posing as erythrocyte PNH clones
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Introduction: Paroxysmal Nocturnal Haemoglobinuria (PNH) is a rare acquired genetic disorder affecting 1-2 people per million. Deletions or mutations in the Phosphatidyl-inositol-glycan-class A (PIG-a) gene causes complete or partial loss of Glycosylphosphatidylinositol (GPI) anchor proteins which serve as protection from the effects of autologous complement activation and haemolysis. Screening by flow cytometry detects the absence of antigens bound to GPI anchors. Loss of antigen expression on both the erythrocytes and leucocytes indicates a PNH clone. Upon performing approximately 1200 PNH screens per year, the laboratory detected 2 cases of CD59 under expression on erythrocytes only, masquerading as a PNH clone. Due to the absence of an abnormality on the leucocytes, neither case fulfilled the criteria to diagnose a true PNH. Further investigations discovered that each case had autoimmune disorders and antiphospholipid antibodies (aPL) were later identified. It is suspected that the presence of aPL may block CD59 expression leading to an ostensible PNH clone being detected by flow cytometry.
Methods: Flow cytometric PNH screening was performed using the BD FACSCanto II. A 5 colour assay was performed to isolate and interrogate monocytes and granulocytes using CD45, CD157, CD15, CD64 and FLAER. CD235a and CD59 are used in the erythrocyte assay and acquired data is analysed using FACSDiva software.
Results: Patient 1 presented with multiple Deep Vein Thrombosis (DVTs) and catastrophic antiphospholipid syndrome (APS). The PNH screen revealed an apparent RBC clone with only 32% expression of CD59, however, the leucocytes showed 100% FLAER expression. This patient went on to have successful treatment for APS and repeat flow cytometry examinations went on to show 100% CD59 expression.
Patient 2 presented with Evans syndrome (immune thrombocytopenia and immune haemolysis), extensive DVTs and thrombophlebitis. Flow cytometry showed reduction in erythrocyte CD59 to a lesser extent of 84% positivity and with 100% positivity for FLAER on leucocytes. Interestingly, this patient also had aPL.
Conclusion: Due to the returning positivity in CD59 on erythrocytes, we conclude it may be possible that aPL (or other autoimmune mechanism) blocks CD59 expression. Its re-expression post treatment, rules out a true CD59 deficiency due to genetic mutation, PNH or otherwise. We aim to highlight the obscurities in detecting true PNH clones with these exceptional events in this rare condition and the need for a broader understanding when screening for PNH by flow cytometry. Additionally, this reiterates the importance of PNH testing in at least two lineages.

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Correlation between multiparameter flow cytometry pattern and BCR molecular breakpoint in acute promyelocytic leukemia - a single institution experience

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Introduction: Acute promyelocytic leukemia (APL) is characterized by a clonal expansion of leukemic blasts blocked at the stage of promyelocytes , characterized by a translocation t(15;17) and a *PML/RARA* gene rearrangement with Bcr1, Bcr2 or Bcr3 breakpoints. APL is a highly aggressive disease requiring rapid diagnosis and early treatment intervention. Multiparametric flow cytometry (MFC) is a rapid technology aiming prompt diagnosis. We therefore conducted analyses of MFC patterns and looked for correlation with BCR molecular breakpoints in APL.
Methods: In this study we analyzed 53 newly diagnosed APL seen at our institution over the past seven years (2008-2014). Among them, 48 were adults and 5 children. In the adult group, 21 patients displayed Bcr1 (43.7%), 3 patients Bcr2 (6.2%) and 24 patients Bcr3 (50%). Subsequently we studied only APL adult patients for Bcr3 vs Bcr1 groups.
Results: Morphological classification showed 71.4% of variant 'microgranular' APL in the Bcr3 group vs 16.6% in the Bcr1 group. Patients were treated according to the APL2006 trial, based on ATRA and chemotherapy as induction therapy allowed CR1 in all patients. Relapses occurred in 4 patients from the Bcr3 group (17.4%) and only in 1 patient from the bcr1 group (5.26%). Six patients died (3 from the Bcr3 and 3 from the Bcr1 groups) very early after diagnosis from coagulopathy. No differences were observed among the two groups in terms of CR, OS or EFS. Leukemia immunophenotyping identified patterns of expression with significant higher expression for CD34 (p=0.004), CD2 (p=0.001), CD56 (p=0.026), CD123 (p=0.01) and CD33 (p=0.02) in the Bcr3 vs Bcr1 group. Moreover, the most immature compartment of leukemia stem cells (LSC) defined as the percentage of CD34+CD38- cells from total leukemic cells was significantly higher in the Bcr3 vs Bcr1 group (p=0.0015). No significant differences were noted regarding CD9, HLADR and the myeloid markers (CD13, CD117, CD15, and CD65).
Conclusions: The retrospective review of histograms in our series confirm previous reports suggesting that immunophenotypic analyses should rapidly identify various patterns of expression with a clear relationship between the Bcr3 group, a pattern combining CD34 + CD38 - CD123 +hi CD2 + CD56 + CD33+hi, and the morphological appearance of more undifferentiated blasts with APL variant 'microgranular' morphology. Our study points especially on the need for quit rapid diagnosis in this type of leukemia, the phenotypic signature should be very useful pending subsequent confirmation with cytogenetic and molecular analysis.

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CD305 (LAIR1) is the most sensitive screening marker for CD5-negative B-lymphoproliferative disorde

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Background: Chronic Lymphocytic Leukemia (CLL) is the most common leukemia in the EU & US but not the most common B-lymphoproliferative disorders (B-LPD). The majority of B-LPD do not express and typically have low levels of blood or bone marrow involvement, therefore the detection of CD5neg B-LPD requires an approach that can distinguish neoplastic cells when they represent a small fraction of total B-cells.
Aim: to determine the most sensitive marker for detection of neoplastic CD5neg B-LPD.
Methods & results: 933 samples (582 CLL/MCL, 326 CD5- B-LPD, 25 polyclonal cases) were assessed for expression of 31 markers. Hierarchical cluster analysis of median fluorescence intensity was performed using dChip software to determine the markers that separated B-LPD from cases with only polyclonal B-cells. Increased CD5 or CD10 and

decreased LAIR1 (CD305) were identified for further evaluation in a screening panel assessing clonality (κ/λ) on gated B-cells (CD19/CD20/CD45) applied to 162 cases of suspected B-LPD (49 CLL/MCL, 32 CD5neg B-LPD, 81 polyclonal). Cases were classified as positive if the κ/λ ratio was >3:1 or <0.3:1 within each B-cell subset (CD5+, CD10+ or CD305wk/neg). CD5 was the most sensitive overall (72% vs. 10% for CD10 vs. 57% for CD305) because most B-LPD assessed were CD5+. For CD5neg B-LPD, LAIR1 was substantially more sensitive than CD10 (69% vs 30%) because CD10+ B-LPD are also consistently LAIR1neg, therefore using LAIR1 permits detection of small sub-populations of germinal centre (GC) B-LPD as well as post-GC B-LPD.
Conclusions: CD305 in substantially better than CD10 for detection of CD5- B-LPD and is recommended in combination with CD5/CD19/CD20/CD45/κ/λ as a screening marker for suspected B-LPD.

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Importance of CD34 in Screening Tests for Hemato-Lymphoid Neoplasms. ClearLLab LS Shows the Way
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Identification of dim CD45 blasts together with exclusion of normal residual cells that follow systematic lineage maturation is crucial in the study of hematopoietic cell differentiation. ClearLLab LS (Lymphoid Screen) is a dried unitized multicolor screening reagent designed for the identification of hemato-lymphoid neoplasms. One of the unique feature of this reagent is the presence of CD34 antibody with the goal to enhance the blast cell identification in supplementation to CD45 expression. CD34 as backbone marker in addition to CD19, CD3, CD10 and others were selected in the panel, owing to its ameliorative expression in a significant number of acute leukemia of any lineage. We undertook the analysis to measure the usefulness of this marker in context of ClearLLab LS reagent to support in diagnosis under the scope of lymphoid screening tube.
Method: A total of 405 clinical specimens from peripheral blood, bone marrow and lymph node samples were collected across Asia, Europe and North American geographies and screened with ClearLLab LS reagent. Data was analyzed to look at the CD34 phenotype and % expression of CD34. The usefulness of CD34 in identifying blast cells were analyzed in the context of other markers and clinical diagnosis. The relative frequency of CD34 expression together with other markers such as CD10, CD5, CD19 and CD56 in Acute B and T and Myeloid leukemia specimens was assessed in all specimens and demonstrated that CD34 in ClearLLab LS reagent resolves blast cells from normal maturing population in acute leukemia of any lineage. Additionally, the evaluation of the patterns of expression of CD10 and CD34 markers in B-ALL specimens demonstrated very clear spatially resolvable partial expression pattern of CD10 and CD34.
Results and Conclusion: Our results demonstrate that CD34 in the ClearLLab LS screening tube augment in identification of blast cells with very good resolution along with other backbone markers in the immunophenotyping of hematological malignancies from normal hematogones in addition to screening of CD34 negative mature lymphoid neoplasms.

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Swaying γδ T Cell Subset Phenotypes Influence HLA B27 Responsive Auto Immunity
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Though CD8 is majorly implicated in immune responses by association with MHC Class I molecule, the functional significance of CD8 homo dimers expressed on VD2 and VD1 subsets of γδ T cells largely remain unknown, since these cells respond largely by MHC independent manner.
HLA-B27 is a MHC I molecule that complexes with β2 microglobulin, thereby, binding short antigenic peptides such as those derived from intracellular microorganisms for presentation to the TCR of CD8+ cytotoxic T cells. Once these complexes are recognized specifically by cytotoxic T cells, they kill the infected cell. Several of the theories contend to establish the relationship between HLA-B27 expression and ankylosing spondylitis and other prominent auto immune disorders like ulcerative colitis, Reiter's syndrome, sacroilitis, uveitis, inflammatory bowel disease. Of the many hypotheses for HLA-B27 mediated auto immune response, the most accepted is the mis-folding of the B27 antigen. This disables pairing of β2 microglobulin, thereby triggering MHC Class I response by cytotoxic T cells .

We undertook studies to compare the varying phenotypes of VD1 and VD2 γδ T cells expressing the CD8 molecule in HLA B27 positive population compared to normal. In addition, functional marker expression profiles between these populations were analyzed and their differential expression patterns and the impact of effector functions of these cell types are discussed in detail.

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Human HLA-B27 Typing Using the BD™ HLA-B27 Kit on the New BD FACSVia™ System: a Multicenter Study

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Introduction: The BD FACSVia™ System* is an easy-to-use flow cytometer that provides workflow efficiency in clinical immunology laboratories. The HLA-B27 application developed on the BD FACSVia System is an algorithm-based assay utilizing the BD™ HLA-B27 kit for routine HLA-B27 screening. In this application, HLA-B27 positive, negative and inconclusive outcomes are reported. To validate performance of the BD HLA-B27 assay on the BD FACSVia System, we conducted an agreement study at three centers using the BD FACSCalibur™ system as a predicate.
Methods: Whole blood samples (n = 594) were stained with BD™ HLA-B27 reagent and prepared using the lyse/wash method. The BD FACSCalibur instrument was set up using BD Calibrite™ Beads. Instrument QC on the BD FACSVia System was performed by running BD™ CS&T Beads. Both cytometer systems were set up for the HLA-B27 assay using BD™ HLA-B27 Setup Beads. Prepared donor samples were acquired on both the BD FACSVia and BD FACSCalibur. The HLA-B27 results reported by the two systems were analyzed to generate overall agreement. HLA-B27 determination of discordant samples was further verified using the Reverse Sequence Specific Oligonucleotide (rSSO) DNA typing method (LABType® SSO, One Lambda).
Results: The BD FACSVia System determined 80 B27+, 499 B27– and 15 inconclusive samples. The BD FACSCalibur determined 73 B27+, 502 B27– and 19 gray zone samples. For the twelve discordant results, one was BD FACSCalibur B27+ vs BD FACSVia inconclusive; eight were BD FACSCalibur gray zone vs BD FACSVia B27+; three were BD FACSCalibur B27– vs BD FACSVia inconclusive.
The overall agreement of HLA-B27+, HLA-B27– and inconclusive samples between the two systems was 98%. None of the HLA-B27 true positive samples reported by the BD FACSCalibur or HLA-B27 molecular typing method was reported negative by the BD FACSVia System. The sensitivity of the BD HLA-B27 assay on the BD FACSVia System was 100%. One in 503 true negative samples was reported as HLA-B27+ by the BD FACSVia System.
Conclusions: The BD HLA-B27 assay on the BD FACSVia System demonstrated robust agreement results compared to the FACSCalibur system, with very high sensitivity. Although a small percentage of samples will be reported as "Inconclusive" and need further verification using other methods, the system provides overall savings in HLA-B27 diagnosis. The automated HLA-B27 application reduces total turn-around time and is suitable for high volume flow cytometry analysis in clinical diagnostic laboratories.
*Product is not yet available for sale in the US.

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Immunophenotyping of Blood Lymphocytes on the New, Easy-to-use BD FACSVia™ System: a Performance Evaluation

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Introduction: Immunophenotyping of blood lymphocytes plays a critical role in the diagnosis, treatment and monitoring of immune system disorders, such as HIV/AIDS, which affects approximately 37 million people around the world including 2.6 million children. To meet global health needs, BD has developed a simple-to-use and affordable flow cytometer, the BD FACSVia System*. It features novel designs in hardware, software and instrument QC that provide workflow efficiencies in clinical laboratories. We evaluated performance of the BD FACSVia System using BD Tritest™ (CD3/CD4/CD45 and CD4/CD8/CD3), and BD Multitest™ (CD3/CD8/CD45/CD4 and CD3/CD16+56/CD45/CD19) kits at two study centers using BD FACSCalibur™ as the predicate system.

Methods: De-identified venous blood specimens (n=165) from HIV+ patients, post bone marrow transplant patients and normal donors were enrolled in the study. Each specimen was stained in four tubes using the two BD Tritest and the two BD Multitest™ reagents in BD Trucount™ Tubes followed by acquisition on the BD FACSVia System and BD FACSCalibur™ instrument, respectively. Both BD FACSVia™ Loader and manual sample loading methods were assessed in the study. Absolute counts and percentages of lymphocyte T, B and NK cells were analyzed statistically using the Deming regression method to compare the test and predicate systems.
Results: For each of the four BD reagents (BD Tritest CD3/CD4/CD45 and CD4/CD8/CD3, and BD Multitest CD3/CD8/CD45/CD4 and CD3/CD16+56/CD45/CD19), results of cell absolute count (Abs) and subset percentages on the BD FACSVia System correlate very well with results obtained on the BD FACSCalibur system. For $AbsCD3^+$, $AbsCD4^+$ and $AbsCD8^+$, 95% confidence interval (CI) of slope ranges from 0.93 to 1.04; R^2 ranges 0.98–0.99. For $AbsCD16+56^+$ and $AbsCD19^+$, 95% CI of slope ranges from 0.85 to 0.96; R^2 ranges 0.95–0.97. For $\%CD3^+$, $\%CD4^+$ and $\%CD8^+$ cells, 95% CI of slope ranges from 0.98 to 1.04; R^2 ranges 0.98–0.99. For $\%CD16+56^+$ and $\%CD19^+$ cells, 95% CI of slope ranges from 0.95 to 1.04; R^2 is 0.98.
Conclusion: The BD FACSVia System reported equivalent results of T, B, and NK subset absolute counts and percentages compared to the predicate BD FACSCalibur system using the two BD Tritest reagents (CD3/CD4/CD45 and CD4/CD8/CD3), and the two BD Multitest reagents (CD3/CD8/CD45/CD4 and CD3/CD16+56/CD45/CD19) with BD Trucount Tubes. The BD FACSVia System is an easy-to-use and affordable system that provides reliable results and simplifies the workflow for human lymphocyte immunophenotyping in clinical settings.
*BD FACSVia System is not yet available for sale in the US.

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Is there a role for flow cytometry immunophenotyping in the study of celiac disease?
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Introduction: Diagnosing celiac disease (CD) is still challenging. Flow cytometry (FC) has contributed to describe a high intraepithelial lymphocyte (IEL) count and an abnormal distribution of IEL subsets. However, updated CD guidelines do not include the use of FC to detect these immunological parameters. We describe our experience on IEL analysis for diagnosing CD and propose a scoring system to facilitate the interpretation of FC data.
Methods: From September 2012 to May 2016, duodenal biopsies from 61 consecutive paediatric patients (<18 years) were sent for FCI analysis. Information from histological lesions, HLA typing, and serum IgA anti-transglutaminase-2 autoantibodies (anti-TG2) were recorded. Clinical data were suggestive of CD in 48 children (median age: 7 years; range: 0.92 to 17). The remaining patients (non-CD cases) were considered controls. After isolation of IELs, cells were stained for 20 minutes at room temperature using a 6-color mAb panel: CD103 FITC/CD56 PE/CD3 PerCP/TCRgd PE-Cy7/CD7 APC/CD45 APC-H7. After one wash, cells were acquired on a FACSCanto II (BDB). The analysis was performed using the Infinicyt software program. Firstly, IELs were identified on the basis of $FSC^{low}/SSC^{low}/CD45^{hi}/CD103^+$, and their percentage related to total cellularity. Secondly, Tgd+ cells ($CD45^{hi}/CD103^+/CD3^+/CD7^+/TCRgd^+$), and NK-like cells ($CD45^{hi}/CD103^+/CD3^-/CD7^+/TCRgd^-$) were identified and their percentage was related to total IEL. Results were expressed as median percentage ± 1SD.
Results: Patients were classified as typical CD (villous atrophy plus increased levels of anti-TG2; n=40) and controversial cases (compatible data for either histology or anti-TG2; n=8). Typical CD showed higher levels of IELS (20.9±13.4%) and Tgd+ cells (30±15%) than controls (5.4±4.4% LIES; 6.8±5.4% Tgd+ cells). Conversely, the levels of NK-like cells were lower in CD patients (3.1±4.6%) than in controls (29.2±19.5%). According to these data and published results, we designed a scoring system: 1 point: >11% IELs, 2 points: >15% Tgd+ cells, 3 points: ≤5% NK-like cells. A score <3 did not indicate CD, a score ≥4 supported CD, and a score =3 was considered indeterminate. Using this FC score, 13/13 negative controls and 36/40 CD patients were correctly classified (specificity 100%; sensitivity 90%, PPV 100%, NPV: 76.5%). The FC score supported the diagnosis of CD in 2/8 children within the controversial group.
Conclusions: In our series, a positive FC result strongly supported the diagnosis of CD. FC data could be of help in cases with discrepancies between histology and serology. Larger studies are warranted to validate the clinical utility of the proposed FC scoring system.

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PNH reticulocytes identification as a fast screening method for PNH-clone evaluation in patients

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Introduction: PNH-clone is rare event so it needs to test many patients with very different diagnosis. International society of clinical cytometry (ICCS) has presented a method for PNH-clone verification which became as a standard for PNH testing. This method is very detailed and includes 8 different reagents with 4 or 6 colors technique. The aim of present work is creation a new method for fast screening on PNH cells. New method had to satisfy main conditions as “one tube implementation”, minimum steps of sample preparation with short time and high sensitivity. Reticulocytes could be those target cells becausethey are not destroying by complement, fast renewal and can be identified simultaneously with erythrocytes in the same tube that gives additional information about PNH-clone in patient.
Methods: To identify PNH reticulocytes we used CD235a+/CD71+/CD59- combination, and for PNH erythrocytes CD235a+/CD59-. Reagents kit was CD235a-FITC (11E4B-7-6), CD59-PE (MEM-43), CD71-APC (OKT9). To identify the sensitivity and quality of new approach comparing with standard method from ICCS both methods were used for every patient. For ICCS method we used CD45-PC7 (J.33), CD235a-FITC, (11E4B-7-6), CD15-PE (80H5), CD64-PC5 (22), CD59-PE (MEM-43), FLAER-AlexaFluorâ 488, CD24-PC5 (ALB9), CD14-PE (RMO52). For erythrocytes analysis we collected no less than 1 million CD235a+cells and for reticulocytes no less than 20 000 CD71+ cells. All measurements were made on FACS Canto II Becton Dickenson cytometer.
Results: 2174 patients with different diagnosis were tested. The number of patient with PNH clone was 439 (113 with minor clone from 0,01% to 0,99% and 326 patients with clone size >1%). Correlation coefficients between clone size on both granulocytes and monocytes with reticulocytes clone size are r=0,921 and r=0,944, correspondingly. This correlation disappeared among patients with PNH-clone less <1%. All patients were double negative or double positive by both methods.
Conclusions: This method allows identify PNH-clone in patients on reticulocytes and erythrocytes simultaneously in one tube without lysis using 3 reagents only. High correlation between PNH-clone size on reticulocytes and leukocytes gives serious evidence of reliability of this method without false negative results. This method can be used for fast screening only and in case when PNH-reticulocytes are found the ICCS method has to be done for leucocytes to get full data about PNH-clone size in patient.

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Simplified Workflow Using Automated Instrument Setup and Compensation on the BD FACSLyric Flow Cytometer

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Introduction: Multi-parameter flow cytometry is used to routinely identify and quantify cell populations in blood and tissue. The BD FACSLyric™ in conjunction with BD FACSuite™ Clinical software, BD™ Cytometer Setup and Tracking (CS&T) beads and dry-format BD™ Fluorescence Control (FC) beads, delivers a seamless and user-friendly workflow from system setup, through assay creation, data acquisition and analysis. The BD FACSLyric system provides new and unique tools to achieve reproducible and consistent setup of assays across time and instruments thereby ensuring reproducible, high performance data.
Methods: To assess accuracy and reproducibility during automated setup, the performance of 3 to six 10-color BD FACSLyric cytometers was measured over 2 months. We evaluated the reproducibility of median fluorescence intensity (MFI) and resolution sensitivity using BD CS&T beads and stained cells, and the reproducibility of spillover with BD FC beads and stained cells. Overall performance was assessed using a multi-color assay for T, B and NK cells.
Results: Three factors determine a flow cytometer’s ability to provide high quality, reproducible data:
1) High detector resolution sensitivity
2) Accurate adjustment of gains to achieve consistent fluorescence targets
3) Accurate adjustment for compensation
The data show that that the BD FACSLyric provides high resolution in all detectors. With the Automated Assay Setup system, a single 20-minute procedure determines gain-independent spillover values (SOV) that are valid for 60 days thus eliminating the need to run daily compensation controls. Variation in the SOVs was typically <0.5%. Similarly, daily setup using CS&T beads (a 5 minute procedure) showed less than 10% variance over time. This

same setup procedure also provided reproducibility of MFIs across six instruments with a variance below 10%. The test multi-color assay also showed excellent reproducibility over time and across instruments.
Conclusions: As flow cytometric technology advances, instrument setup and quality control (QC) have become cumbersome and time consuming. The data presented here demonstrate that the BD FACSLyric platform delivers user-friendly features such as automated instrument setup and compensation while achieving reproducible and consistent results. The same user-friendly features result in time savings every day. Also, instrument-to-instrument standardization ensures that results can be shared across sites.

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Analysis of Checkpoint Marker Expression on Immune Cells Using a 10-Color Assay on The BD FACSLyric
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Introduction: The immune system plays a critical role in cancer progression. Recently, some patients with melanoma have shown a beneficial therapeutic response to immune checkpoint-inhibiting drugs. Much work is being done to explore the power of immune checkpoint inhibitors for treatment of additional cancer types. Immune checkpoint markers include programmed cell death-1 (PD-1 i.e. CD279) and its ligand PDL-1 (CD274); the proliferation modulator CD152; the costimulatory molecules CD137, CD134, CD28 and its ligand CD86. In designing therapeutics that exploit these markers, an accurate means of measuring degrees of expression is critical to patient selection and therapy evaluation. Here we demonstrate an elegant 10-color flow cytometry assay aimed at the quantitation of immune checkpoint markers on peripheral blood mononuclear cells (PBMC) following ex vivo stimulation.
Methods: Immune checkpoint surface expression was evaluated using a 10-color flow cytometry panel and the BD FACSLyric™ platform. PBMCs were cultured with and without stimulation using a variety of stimulatory agents or cytokines. The kinetics of immune checkpoint marker expression were also explored.
Results: Cultured PBMCs exhibited robust increases in immune checkpoint marker expression levels in both the T-cell compartment and the non T-cell compartment following stimulation. The observed increases in checkpoint marker expression were both time- and concentration-dependent and also followed predictable patterns based on the known qualities of each stimulatory agent.
Conclusions: The utilization of a 10-color flow cytometry assay on the BD FACSLyric flow cytometer demonstrates an accurate and reproducible method of determining expression levels of immune checkpoint markers. The expression of these immune checkpoint markers is an inducible phenomenon that can be achieved by simulating the conditions of the tumor microenvironment and can be controlled through culture conditions and by the choice of stimulatory agent employed. Assessment of immune checkpoint markers may provide insights to help inform the patient selection process, to help monitor treatment effectiveness, or to help design or optimize more effective immunomodulatory drugs.

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Enhanced Reproducibility of Multi-color B-Cell Assays Using Automated Universal Assay Setup Features of the BD FACSLyric and Dry-Format Reagent Panels
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Introduction: Accurate and reproducible assays are the cornerstone to meaningful results from multi-site clinical trials. Two of the major sources of variability in such studies are 1) setup and cross-site standardization of instruments and 2) reagent consistency. Data presented here show that the Automated Universal Assay Setup functionality of the BD FACSLyric™ cytometer / BD FACSuite™ system which leverages BD™ CS&T and BD™ FC Beads provides the necessary instrument setup as well as enhanced assay portability. However, even with standardized instrumentation, variations in reagent panels over time is the most common source of variability in results from lab developed assays. BD Life Sciences has developed multi-color dry format cocktails which minimize assay variations and provide long-term reagent stability to further enhance assay reproducibility. Together these features provide consistency in assay performance across multiple instruments
Methods: Two B-Cell panels (P1= CD19, CD20, CD22, kappa, lambda, CD45, CD5, CD10; P2= CD19, CD22, CD79b, CD23, CD5, CD11c, CD45) designed by the Tex Flow consortium to enumerate a variety of sub-populations were dried to stabilize the reagent cocktail. Assay performance was evaluated using three BD FACSLyric platforms using the BD FACSuite Universal Setup. System spillover values for compensation were applied and no manual compensation was required. The assays were run using the same donors on three instruments over a two month

timeframe.
Results: Results were acquired across three instruments over a two month timeframe. The assays delivered excellent resolution for all parameters with accurate compensation. Data was collected in the absence of daily compensation controls or any manual adjustment, spillover values typically showed less than 0.5% difference over time. Analysis of the median fluorescence (MFI) reproducibility of individual populations across instruments, a key requirement for cross-site assay portability and reproducibility, showed variances between 2% and 11% depending upon the detector. When looking over time similar MFI variances were seen albeit slightly larger due to donor differences. Not surprisingly given this reproducibility the data show that uniform gating across instruments and times provided consistent results in terms of identification and quantification of individual sub-populations.
Conclusions: Dried reagents stabilize assay performance over time, while the enhanced features of the BD FACSLyric enable the use of Universal Setup to deliver equivalent results when tested using three platforms. These features enable standardization of assay results simplifying data comparison of the LDA delivering equivalent performance over time and across instruments.

52 (Selected Best Poster Abstract Presentation)

Analysis of immune infiltration assess by multiparametric flow citometry in brain tumors: characterization of myeloid subpopulation cells

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Introduction: The central nervous system (CNS) can be affected by different types of neoplasms with variable prognosis. Meningiomas are considered the vast majority to be benign and slow-growing neoplastic lesions. Conversely, gliomas are malignant tumors from which glioblastoma (GBM) is the most common and aggressive primary brain tumor in adults with a median survival of only 12 months. In spite of the wide knowledge about the great clinical heterogeneity, histopathology, higher recurrence rates, clinical aggressiveness, and outcome of all these tumors, the immune mechanisms that would be on the basis of the origin and progression of these tumors are still unknown. For this reason, our aim was the characterization of immune cell infiltrates with special attention to myeloid cells in central nervous system (CNS) tumors by flow citometry.
Methods: Meningioma WHO grade I (n=19) and GBM (n=11) samples were obtained from patients diagnosed with undergoing surgery at the University Hospital of Salamanca. A portion of fresh tumor sample was mechanically disaggregated and stained with the following panel of monoclonal antibodies: CD45-OC515, HLADR-BV711/HLADR-PB, CD14-BV650, CD33-PE-CFS594, CD11B-FITC, CD16-BV786, CD123-PE, CD44-PE, CD192-BV421, and CD206-FITC. A live DNA-stain (DRAQ5) was applied as a viability marker. Samples were acquired in a Fortessa flow cytometer and analyzed with Infinicyt software program.
Results: Similar amount of infiltration was found in both, GBM (median:28%; range: 10%-65%) and meningioma (median: 23%; range: 3%-76%) tumors, being more variable in the last group. Lymphocytes, neutrophils, antigen presenting cells (APCs) and myeloid cells without presenting antigens ability were identified. Lymphocyte population showed significant differences, being double in meningiomas (12%) than GBM (6%). Moreover, population of neutrophil represented 11% in GBM versus 1% in meningiomas. Other significant differences were found in the APCs population that consisted in 97% of myeloid cells in meningiomas versus 70% in GBM. Inside this group, different expression for each marker allowed to identify macrophage subset losing CD14. Furthermore, a group of $HLADR^-$ myeloid cells ($CD11B^+$, $CD33^+$ $HLADR^-$) constituted the 8% in GBM versus 1% in meningiomas.
Conclusions: The higher content of APCs and lymphocytes found in meningiomas suggest a good response of the immune system compared with GBM tumors in which neutrophils and $HLADR^-$ myeloid cell populations (possible myeloid derived suppressor cells), could be related with the malignancy of these tumors and their poor prognosis. Thus, the need of a deep characterization of these populations is imposed.

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Viable CD34+cell enumeration in the auto- and allo-transplant settings with ISHAGE technology: Updates for Navios and Canto cytometers

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Background: Enumerating CD34+ cells provides critical information to the bone marrow transplant physician. The number of viable CD34+ cells present in the peripheral blood after mobilization predicts the 'yield' of CD34+ cells in the apheresis product. The infusion of a minimum of 2-2.5 x 10^6 viable CD34+ cells per kilogram patient weight will generally ensure rapid (10-12 days for neutrophils to 500/uL) and sustained engraftment in the autotransplant setting.
Methods: The single platform ISHAGE protocol that utilyzes CD45FITC, CD34PE, 7-AAD (a viability dye) and fluorescent counting beads is the most widely used method to count viable CD34+ cells in clinical laboratories and several commercial 'kits' are available based upon the ISHAGE Guidelines (for auto transplants). Manual data acquisition/analysis templates/protocols were developed for a variety of older cytometers equipped with 4 (Calibur, BD Biosciences) or 5 (FC500, Beckman Coulter) fluorescence defectors (PMTs). The widespread deployment of 6/8 PMT Cantos (BD Biosciences) and 8/10 PMT Navios (Beckman) platforms required the development of equivalent assays that could run on instruments running modern operating systems/cytometry software.
Results: We validated the single platform ISHAGE protocols across instruments with 4 PMTs (Calibur), 5 PMTs (FC500), 8 PMTs (Canto II), and 10 PMTs (Navios) and show that equivalent data is generated regardless of instrument platform/software combination used (T-Test showed no significant difference). Analysis of 20 fresh mobilized PB/apheresis samples across FC500, Navios, Calibur and Canto instruments yielded virtually identical results with high correlation coefficients (0.999). The addition of a CD3PCy7 conjugate (Beckman) or CD3APC conjugate (BD) allowed the development of 4-color variants of the single platform ISHAGE methodology that can simultaneously measure not just absolute viable CD34+ and CD45+ cell content but also the absolute viable CD3+ cell content. This assay has utility in the context of graft assessment in the allotransplant/matched unrelated donor setting in which samples often need to be shipped over long distances. FC500-, Navios-, Calibur- and Canto-specific versions were developed and analysis of fresh samples again yielded virtually identical results with high correlation coefficients (0.99 or better).
Conclusions: We have validated the auto and allo variants of the single platform ISHAGE protocol across multiple platforms. The allo variant can also be used to measure the CD34+ cell purity and residual contaminating viable CD3+ cells in CD34+ cell-selected samples. The allo variant can also be used to accurately determine the number of viable CD3+ cells when donor lymphocyte infusion is required post transplant.

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Demonstration of the Clinical Performance of the ClearLLab LS in a Multi-Center Study
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Introduction: ClearLLab Lymphoid Screening (ClearLLab LS*) is a multicolor leukemia and lymphoma reagent panel for immunophenotyping of normal and abnormal hematolymphoid cells in peripheral whole blood (WB) or bone marrow (BM) collected in K_2EDTA, heparin or ACD anticoagulants, or lymph node (LN) tissues. The reagent consists of 10 fluorochrome and 12 antibodies (against T, B and NK lineages) in a dried format in one tube.
Methods: The clinical accuracy of ClearLLab LS was evaluated by comparing the agreement of the LS panel to the existing CE-marked comparator reagent (ClearLLab**) for detecting the presence or absence of abnormal populations in specimens evaluated by flow cytometry for suspicion of leukemia and lymphoma. The targeted subjects were based on 2006 Bethesda International Consensus Recommendations, and only specimens from new cases or follow-up for lymphoid neoplasms were included. As secondary endpoint, the study also evaluated the ability of the ClearLLab LS to correctly assess the maturity of the abnormal populations as compared to the predicate method. Subjects were enrolled from 4 clinical sites across the United States, Canada and Europe. The principal investigators (PIs) at the sites were blinded to the diagnosis and assessed phenotype results for ClearLLab LS and ClearLLab independently.
Results: A total of 210 subjects were enrolled (102 WB, 77 BM and 31 LN specimens) and 118 of them were clinically diagnosed with hematologic malignancy, including 80 B-cell malignancies, 11 T/NK malignancies and 27 myeloid malignancies. Our preliminary analysis indicates that the ClearLLab LS agreed 100% with ClearLLab in excluding the presence of an abnormal phenotype, and 99.1% agreement in detecting the presence of abnormality, with an overall agreement of 99.5%. Additionally, the two reagent panels had a 100% agreement for

the designation of maturity of the abnormal populations.
Conclusions: The ClearLLab LS reagent panel showed good phenotype agreement with the ClearLLab in assessing the presence or absence of abnormal populations.
**ClearLLab LS is in development, pending achievement of CE compliance; not available for in vitro diagnostic use*
***ClearLLab is CE marked but may not be available in all markets.*

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Distribution of T-cell receptor Vβ repertoire usage in ten T-cell subtypes in peripheral blood of healthy adults from India
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Introduction: Flow cytometric (FC) analysis of T-cell receptorVβ repertoire (TCR-Vβ-R) is a well-established technique of T-cell clonality determination for the diagnosis and monitoring of T-cell lymphoma. It has been also used for studying selective T-cell responses in different cellular immune diseases. For correct interpretation of these studies, we need information about TCR-Vβ-R usage in healthy individuals. Till date, the levels of TCR-Vβ-R usage in healthy individuals are reported only in CD3+T-cells, CD4+T-cells and CD8+T-cells. However, TCR-Vβ-R usage in the other subsets of T-cells is totally unknown. Hence, we studied the normal levels of TCR-Vβ-R usage in ten immunophenotypic subsets of T-cells in healthy individuals from India.
Methods: This prospective study was carried out in Tata Memorial Hospital, Mumbai. Distribution of TCR-Vβ-R expression levels was studied in 100 healthy adults using Beckman Coulter's TCR-Vβ-R kit that includes antibodies against 24 commonly expressed TCR-Vβ-proteins. TCR-Vβ-R was studied using 8-color FC-immunophenotyping on Navios flow-cytometer and data was analyzed using Kaluza-software. Informed consent was obtained from each participant as per Institutional Ethics Committee.
Results: Peripheral-blood samples of 100 healthy participants (age between 18-70 years & male-42, female-58) were studied to determine the expression levels of twenty-four TCR-Vβ proteins by various subsets of CD3+T cells that included CD3+ T-cells, CD3+4+ T-cells, CD3+8+ T-cells, CD3+ (CD4/CD8 double+) T-cells, CD3+ (CD4/CD8 double-) T-cells, CD7-CD4+T-cells, CD7-CD8+T-cells, CD7- (CD4/CD8double-)T-cells, CD16+CD56+CD4+T-cells and CD16+CD56+CD8+T-cells. Distribution of 24 TCR-Vβ protein expression levels was determined and their normal ranges, mean, median, standard deviation (SD) and CV were calculated. Mean/SD values for total of 24-Vβ-protein usage by these T-cell-subsets were as follows- CD3+T-cells: 62.11%/5.69%, CD3+4+ T-cells: 61.54%/6.19%, CD3+8+ T-cells: 56.67%/8.62%, CD3+(CD4/CD8 double+) T-cells: 80.16%/22.86%, CD3+(CD4/CD8 double-) T-cells: 61.72%/11.48%, CD7-CD4+T-cells:61.47%/10.13%, CD7-CD8+T-cells: 56.39%/16.55%, CD7- (CD4/CD8double-) T-cells: 57.74%/13.93%, CD16+CD56+CD4+ T-cells: 76.48%/21.51% and CD16+CD56+CD8+T-cells: 63.48%/17.19%. Due to the word limitations, results of individual Vβ-protein usage by each subset could not be included in the abstract.
Conclusion: Our study first-time elaborated the distribution of T-cell-receptor-Vβ repertoire usage in ten subtypes of T-cells in healthy adults. These values can be used for correct interpretation of clonality assessment in T-cell neoplasms expressing immunophenotype similar to anyone of these subtypes, especially in samples with low proportion of tumor-cells in the background of normal T-cells.

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Performance Evaluation in Europe of the BD FACSLyric™ 10-Color System Using Remnant Specimens with BD Tritest™ Reagents
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The BD FACSLyric™ system consists of a flow cytometer - available in different optical configurations, the BD FACSuite™ Clinical software, the optional BD FACS™ Universal Loader, and the optional BD FACSLink™ interface for data transfer to a Lab Information System (LIS). The BD FACSuite Clinical software, used with BD™ FC beads and BD™ CS&T beads, supports IVD universal setup (performance QC, instrument control), data acquisition and storage, on/off-line data analysis.
A performance evaluation was conducted with the BD FACSLyric 10-color configuration using de-identified and

delinked remnant venous specimens from HIV-infected and uninfected patients. Samples were tested in the predicate systems (BD FACSCalibur™ with BD Tritest™ CD3/CD4/CD45 and CD4/CD8/CD3 reagents, BD Trucount™ tubes, BD Trucount™ controls) for comparison with the BD FACSLyric system.
The BD Tritest reagents used for this evaluation were BD Tritest CD3/CD4/CD45 (N = 106) and BD Tritest CD4/CD8/CD3 (N = 121). For analysis, the percent mean biases of the absolute counts/µL and percentage of lymphocytes were calculated for the different lymphocyte subsets and per BD Tritest reagent, as shown. Deming regression results gave R^2 ≥0.96, and slope estimated values were between 0.99 and 1.04.
In conclusion, the performance of the BD FACSLyric flow cytometer with BD FACSuite Clinical software was equivalent to the BD FACSCalibur flow cytometer with BD Multiset™ software predicate system using the BD Tritest CD3/CD4/CD45 and BD Tritest CD4/CD8/CD3 assays. The study was sponsored by BD Biosciences.

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Performance Evaluation of the BD FACSLyric™ 10-Color System with BD Multitest™ Reagents using Remnant Specimens

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The BD FACSLyric™ system consists of a flow cytometer - available in different optical configurations, the BD FACSuite™ Clinical software, the optional BD FACS™ Universal Loader, and the optional BD FACSLink™ interface for data transfer to a Lab Information System (LIS). The BD FACSuite Clinical software, used with BD™ FC beads and BD™ CS&T beads, supports IVD universal setup (performance QC, instrument control), data acquisition and storage, on/off-line data analysis.
A performance evaluation was conducted with the BD FACSLyric 10-color configuration using delinked and de-identified remnant whole blood from HIV-infected and uninfected patients. Remnant samples were prepared following the manufacturer's instructions for the BD Multitest™ IMK kit and 6-color TBNK reagents with BD Trucount™ tubes, and tested using the BD FACSLyric system and the predicate system (BD FACSCanto™ II (4-2-2) with BD FACSCanto™ clinical software. The 6-color TBNK (N=110) was analyzed for percent mean biases for the absolute counts/µL and percentage of lymphocytes. Deming regression gave an R^2 ≥0.95, and slope estimated values were between 0.98 and 1.08.
Similar analyses were performed for the IMK kit N=121). The absolute counts and percentage of lymphocytes percent mean biases were calculated for CD3 average, CD3-tube-1, CD3-tube-2, CD4, CD8, CD16+CD56, and CD19. Deming regression gave an R^2 ≥0.98. The slope estimated values were between 1.00 and 1.05.
In conclusion, the performance of BD FACSLyric with BD FACSuite Clinical software was equivalent to the BD FACSCanto II with BD FACSCanto clinical software system using BD Multitest assays. The study was sponsored by BD Biosciences.

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A validation of 10-color flow cytometry tube in body cavity fluids

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Introduction: Cytomorphology evaluation of cellular components in body cavity fluids (BCF) analysis has been used as gold standard. However, morphologic distinction has variability between technicians, especially in hypocelular and bloody specimens. Manual leukocyte differential count (LDC) needs extensive and continue training resulting in time consuming. The aim of this study was to design a 10-color flow cytometry tube to compare with cytomorphology and a haematology analyzer (Sysmex-XN9000) in BCF LDC.
Methods: We evaluated 10 samples of BCF (4 ascitic/6 pleural). The samples were stained according to a designed 10-color panel (CD4+Kappa FITC/CD8+LAMBDA PE/CD3+CD14 ECD/CD33 PC5.5/CD19+CD56 PC7/CD34 APC/CD71 AP700/CD38 APC-H7/CD20 PB/CD45 KO). Acquisition was in Navios Flow Cytometer (Beckman Coulter)(BC), analysed in Kaluza (BC) and Infinicity (Cytognos). Comparison was with manual LDC by cytospin slide with panchromatic stain and XN9000. The nucleated cells were separated in mononuclear (MN), polymorphonuclear (PMN) and other nucleated cells like mesothelial, epithelial and non-haematologic tumours cells (NHTc).

Results: We compared the flow cytometry (FC) LDC with cytomorphology (reference method) and in all samples, the correlation test was r^2=0,92 to MN and r^2=0,89 to PMN. However, one sample showed suspicion of NHTc by cytomorphology while FC did not detect tumour cells. Absence of malignancy was confirmed by Anatomical Pathology. FC LDC and XN9000 correlation test was r^2=0,94 to MN and r^2=0,97 to PMN. The mean of acquired cells was 39.800 of total events.
Conclusion: The importance of LDC in BCF is to identify whether cells are predominantly polymorphonuclear or mononuclear. This is crucial to help the separation of infection from other pathologies and allows the clinician to start empirical treatment as soon as possible. The designed 10-color tube was capable to identify T-lymphocytes (subpopulations), B-lymphocytes, monoclonality, monocytes/macrophages and neutrophils also could be used to identified haematological neoplasms, plasma cells, progenitor cells and helps NHTc suspicious samples (CD45-/CD56+). Despite of the good correlation of MN and PMN between cytomorphology and FC, the morphology analysis could detect mesothelial cells better than FC due to absence of specific marker for these cells in our tube. The correlation between XN9000 and FC was good, but the haematology analyzer could not differentiate mesothelial cells, macrophages or NHTc while FC could detect and differentiate monocytes/macrophages from mesothelial and NHTc. The designed tube is reproducible, less subjective, faster in BCF analysis and several thousand cells are counted. Our next challenge is to evaluate mesothelial and epithelial cells markers to improve our validation panel.

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Th1 and Th17 polarizing capacity of peripheral blood dendritic cells in psoriasis
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Activation of T lymphocytes in psoriatic skin lesions is an important immunological mechanism of pathogenesis of the disease. Within psoriatic plaques there are large numbers of dermal dendritic cells and they play a crucial role in the activation and polarization of T cells. Characteristics of skin DCs in psoriasis are well defined, however, the involvement of their peripheral blood precursors in disease pathogenesis is poorly understood. Therefore, we aimed to characterise the different DC subpopulations separated from the blood of patients with psoriasis, to examine their phenotypical features, their intracellular cytokine and chemokine production. Peripheral blood myeloid and plasmacytoid DCs (pDCs) were separated from the blood of psoriasis patients and healthy controls using Blood Dendritic cell magnetic separation kit. Cell surface markers and intracytoplasmic cytokine production were investigated with flow cytometry applying 8-colour staining. With this methodology we could simultaneously investigate their T cell polarising capacity with the examination of IL-12 as Th1, IL-2 and CCL17 as Th2, TGFβ, IL-23, IL-6 as Th17, IL-6 and TNFα as Th22, and TGFβ and IL-10 as Treg polarizing cytokines. According to our results both psoriatic and control DCs are able to produce IL-12 (as Th1 polarizing cytokine), but psoriatic DCs produced it in higher amount. Among the examined DC populations pDCs were found to participate in the production of other psoriasis related cytokines too, since beside IL-12 they also produced IL-23 and IL-6 (as Th17 polarizing cytokines) in significantly higher amount compared to healthy controls. When examining the chemokine production of the DCs we have found that both psoriatic and healthy DCs produced CCL3, CXCL1 chemokines. Our results indicate that beside skin DCs, circulating pre-DCs deriving from psoriatic patients are also able to produce psoriasis specific cytokines and they are characterized by a Th1 and Th17 polarizing capacity.

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Analysis of T and B cell subsets in healthy subjects and its implication in common variable immunodeficiency monitoring
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Introduction: Common variable immunodeficiency (CVID) is a heterogeneous disease with different causes of hypogammaglobulinemia. Because CVID symptoms are unspecific and extremely variable, diagnosis of CVID can be challenging. Therefore, reliable normal healthy control baseline values are indispensable. The use of domestic reference values is known to improve the accuracy of flow cytometric analysis by integrating local variation due to race, gender, and age. Here we report establishment of reference values for a wide range of peripheral blood lymphocyte phenotypes that apply to healthy adult population in Slovenia.
Methods: The study was done on 26 healthy adult volunteers in comparison to 9 well-characterized CVID patients. A quantitative multiparametric flow cytometry was used to estimate absolute and proportional values for a range of T-, B-, and NK-cell subsets. Data are expressed as median together with percentile P5 and P95.

Results: Reference values were obtained for the absolute and proportional levels of total CD3+ T cells (1035-2356 cells/microL, 61,0-81,6%), helper CD4+ T cells (622-1,470 cells/microL, 31,0-55,0%), cytotoxic CD8+ T cells (248-1010 cells/microL, 14,5-55,7%), activated CD4+ T cells expressing CD25 (26-446 cells/microL, 9,5-18,1%), regulatory T cells expressing CD25highCD127lowCD4+ (11-93 cells/microL, 4,1-8,8%), activated T cells expressing HLA-DR+CD3+ (58-347 cells/microL, 4,6-21,3%), CD3+CD4-CD8-TCRab DNT alpha beta cells (12-153 cells/microL, 0,7-7,2%), CD3+CD4-CD8-TCRgd DNT gamma delta cells (4-221 cells/microL, 0,4-13,5%), Recent Thymic Emigrants = CD31+CD3+CD4+ (131-697 cells/microL, 20,3-55,1%), naive Th cells =CD45RA+CD4+ (218-945 cells/microL, 31,4-68,9%), memory Th cells = CD45RO+CD4+ = (272-827 cells/microL, 31,1-68,6%), CD4+IFNg+ = Th1 (46-274 cells/microL, 4,5-20,4%), CD4+IL-4+ = Th2 (4-42 cells/microL, 0,4-5,0%), CD4+IL-17+ = Th17 (2-17 cells/microL, 0,2-1,6%), CD19+ B cells (117-449 cells/microL, 5,6-13,8%), total memory B cells naive = CD19+CD27+ (9-125 cells/microL, 5,2-41,0%), nonswitched memory B cells = CD19+CD27+IgD+IgM+ (3-73 cells/microL, 2,1-24,3%), IgM-only memory B cells = CD19+ CD27+ IgD- IgM+ (0-5 cells/microL, 0,2-8,6%), class-switched memory B cells = CD19+ CD27+ IgD- IgM- (3-62 cells/microL, 1,6-21,9%), immature B cells = CD19+CD21lowCD38low (4-24 cells/microL, 1,9-9,2%), mature B cells = CD19+ CD21+ (110-436 cells/microL, 88,4-97,2%), IgM B cells = CD19+ IgM+(6-119 cells/microL, 5-32,9%), transitional B cells =CD19+ CD38+ IgM+(2-36 cells/microL, 1,2-8,9%), plasmablasts =CD19+ CD38+ IgM- (5-37 cells/microL, 2,6-9,5%), NK-cells = CD3(-)/(CD16(+)/CD56(+)) (136-613 cells/microL, 6,5-26,1%).
Conclusion: With this localized reference values for an extended range of peripheral blood lymphocyte subsets we successfully classified nine CVID patients and we hope to enhance the utility of flow cytometric analysis undertaken in Slovenia.

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A real-time flow cytometry assay of the activity of Na+/H+ exchanger in whole-blood leucocytes

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Introduction: The Na^+/H^+ exchanger (NHE) is an integral membrane protein that extrudes H^+ in exchange for extracellular Na^+ and plays a crucial role in the regulation of intracellular pH (pHi). NHE is critical for the maintenance of pHi during ischemia,but there is growing evidence of a pivotal role for NHE in tissue injury during ischemia and reperfusion. We had previously set up (Dolz M et al. Cytometry A. 2004; 61:99) and validated (Lequerica JL et al. J Physiol Biochem. 2006; 62: 253) a flow cytometry kinetic method to measure the activity of NHE in different cell lines. Here, we present an adaptation to determine NHE activity in leucocytes from human whole blood samples.
Methods: 25 mL-samples of human whole blood were incubated with 5 mL of CD45-PECy5 antibody for 15 min and diluted to 1 mL with bicarbonate-free RPMI 1640 medium containing 25 mM HEPES, to abolish the activity of other cellular pH-restoring systems. Then, 5 mL of pH-indicator BCECF-AM (1 mM) were added and the sample incubated for 15 min at 37º C in the dark with or without 4 mM ethyl-isopropyl-amyloride (EIPA). Samples were run on an Accuri C6 or a Gallios flow cytometers and the following parameters acquired: FS, SS, CD45-PECy5 red fluorescence, BCECF fluorescences at 525 nm (FL1) and 620 nm (FL2) and the Ratio FL2/FL1 (an estimation of pHi) and Time (up to 300 s). Sample was run for 10 s, then paused, and 50 mL of 1 M sodium propionate, pH 7.4 (ProNa) added. Data acquisition was re-started to show induced acidification as a decrease in the ratio FL2/FL1.
Results: In whole-blood unlysed samples gating on CD45 showed clearly leukocyte subpopulations. Analysis of pHi showed that leukocyte subpopulations differed in resting pHi, granulocytes being more alkaline. Kinetic analysis of pHi following intracellular acidification induced by ProNa showed heterogeneity in WBC responses. Selection of specific subpopulations on SS vs CD45 expression showed that lymphocytes do not recover pHi, whereas monocytes and granulocytes return rapidly to resting pHi. The participation of NHE is confirmed by the inhibitory effect of EIPA in all leucocytes.
Conclusions: This is the first real-time cytometric assay measuring NHE responses to intracellular acidosis in leucocytes under physiological conditions. This assay may be used for pharmacological studies of NHE, a therapeutic target for conditions in which ischemia-derived acidosis is involved. Sponsored by UVEG (UV-INV-AE15-349700)

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Characteristics of peripheral blood natural killer cells in women with recurrent miscarriages

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Introduction: Approximately 2-5% of couples may suffer from infertility due to spontaneous abortion. Recurrent miscarriage (RM) is usually defined as two or more consecutive pregnancy losses. RM is influenced by hormonal balance, chromosomal abnormalities, uterine abnormalities, or immunologic function. Immunologic responses in women with RM have been evaluated by measuring different lymphocyte subsets. Natural killer (NK) cells play an important role in female reproductive performance and have been thought to be associated with embryo implantation, RM or infertility. In addition to NK cell, other lymphocyte subsets could be the immunologic factor attributed to RM. The aims of this study were to compare the percentage of peripheral blood lymphocyte subpopulations in patients with recurrent miscarriages and patients without abortion history and to compare the NK cells according to mean fluorescence intensity (MFI).
Methods: The patient group included 11 women with recurrent miscarriages. Recurrent miscarriages were defined as at least two consecutive idiopathic spontaneous pregnancy losses before 22 weeks of gestation. The control group comprised 6 patients without recurrent miscarriages. The percentages of lymphocyte subsets were determined in peripheral blood (PB) using a standard single-platform technique, the TetraONE System (Beckman Coulter, Miami, USA). Four-antibody-fluorochrome combinations (anti-CD45-FITC/anti-CD56-PE/anti-CD19-ECD/anti-CD3-PC5) were used during immunofluorescence analysis. The sample acquisition and flow cytometric immunophenotypic analysis were performed on the flowcytometer, Cytomics (Beckman Coulter), with a fully automated software-reagent combination. Cell subsets were evaluated based on the expression of $CD3^{+}CD56^{+}$, $CD3^{+}CD56^{-}$, $CD19^{+}$, $CD3^{-}CD56^{+}$ ($CD56^{dim}$, $CD56^{intermediate}$, $CD56^{bright}$). The data are presented as median (range). Mann-Whitney *U* test was used to compare the percentages of cells between groups.
Results: The median percentages (range) of $CD56^{dim}$ cells were 16.25 (8.26-25.84) for patient group, and 9.69 (6.17-13.12) for control group, respectively (*P*=0.027). The percentages of $CD56^{bright}$ cells were 0.22 (0.02-0.30) for patient group, and 0.17 (0.13-0.96) for control group (*P*=0.660). The percentages of NKT cells ($CD3^{+}CD56^{+}$ cells) and B cells ($CD19^{+}$ cells) were not significantly different in patient group compared with those with control group.
Conclusion: Increases in the percentage of $CD56^{dim}$ cells in peripheral blood may be important immunologic factors for RM. Measurement of NK cells in the PB may be a useful tool for predicting patients' risk of RM and planning therapeutic plan.

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Regulatory T cells and cells producing cytokines in children with primary hypertension

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Introduction: The aim of the study was to find out if the peripheral blood Treg population level and the levels of effector and proinflammatory cytokines producing T cells differ between the primary hypertension children (PH, n=58), and normotensive control (n=22). Based on the expression of adiponectin receptor AdipoR1 the patients were divided into two groups: A: high expression [AdipoR(+)] and B: low expression [AdipoR(-)] of the test marker on leukocytes (neutrophils and monocytes).
Methods: T-cell subtypes were defined by cell surface markers and intracellular cytokines (IL-17,IFN-g,TNF-a). For enumeration of Tregs (CD4+/CD25+/CD127-), T effector (CD4+/CD25-/CD127-) and cytokines-producing cells we used conjugated monoclonal antibodies in direct method. The leukocyte adiponectin receptor expressions (AdipoR1) were determined with the use of direct and indirect three-color flow cytometry method. Serum adiponectin concentration was measured by ELISA method.
Results: The group of 27 AdipoR1(-) PH patients showed a significantly greater percentage of Treg (9.45±1.81) than 31 AdipoR1(+) children (7.35±1.22) and 22 normotensive ones (7.45±1.77) (P<0.001). There weren't statistically significant differences in the percentage of Treg cells between the AdipoR1(+) group and the control (P=0.80). We also observe significantly higher percentage of cells producing proinflammatory cytokines ($IL\text{-}17^{+}$,$IFN\text{-}g^{+}$ cells) in patients compared to the control group (n=17, 1.58±0.7, 2.23±0.84 respectively, P<0.01). The highest proportion of proinflammatory cells we observe in B group (n=20, $IL\text{-}17^{+}$: 4.76±1.77, $IFN\text{-}g^{+}$: 4.25±2.06), even in comparison with the A group (n=24, $IL\text{-}17^{+}$: 2.47±0.81, $IFN\text{-}g^{+}$: 3.20±1.24) of PH patients (in all cases P< 0.001). The percentage of cells producing TNF-a significantly increase in both groups of patients (A group: n= 24, 3.07±0.94, B group: n=20, 3.78±2.28) compared to the control (n=17, 2.29±1.16)(P< 0.05). Importantly, neutrophil but not

monocyte AdipoR1 expression levels in the PH children was inversely related both to the Tregs (r=-0.45; P<0.0001) and IL-17 (r=-0.50; P<0.0001) or IFN-g (r=-0.30; P=0.04) producing CD4+ T cell numbers. In contrast, monocyte AdipoR1 expression levels were negatively correlated only to the numbers of IL-17 (r=-0.34, P=0.004) and IFN-g (r=-0.32, P=0.027) bearing CD4+ T cells. Low leukocyte AipoR1 expression was not associated with serum adiponectin levels but increased leukocyte AdipoR1 expression was related to decreased serum adiponectin concentrations.
Conclusions: Neutrophil AdipoR1 up-regulation, possibly associated with low adiponectin levels, may participate in down regulation of Treg subset and increase in proinflammatory responses, manifested here by elevation of proinflammatory IL-17 and IFN-g bearing T cell numbers. The study was supported by NSC 2013/11/B/NZ4/03832.

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Regulatory cells, Th17 and dendritic cells in the development and control of H. pylori-induced inflammation in children

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Introduction: Dendritic cells (DCs), regulatory T cells (Tregs) and IL-17-producing helper T cells (Th17) play an important role in the regulation of inflammation.
The aim of this study was to investigate: 1) the peripheral blood distribution ofTregs, Th17 cells, plasmacytoid (pDCs) and myeloid (mDCs) DC subsets, and 2) the activation status of DCs in *Helicobacter pylori (H. pylori)*-infected children, with the goal of comparing the results with those obtained from the controls. Additionally, the presence and localization of these cellsin gastric mucosa will be assessed.
Methods: Thirty-six children were studied: twenty-one of them with *H. pylori*. *H. pylori* infection status was assessed based on [^{13}C] urea breath test, rapid urease test, and histology. Peripheral blood and gastric antral biopsy specimens were collected from patients undergoing endoscopic examination. The frequencies of Tregs with the chemokine receptor CCR6 (identified as $CD4^+CD25^{high}FoxP3^+CCR6^+$), Th17 cells ($CD4^+IL\text{-}17^+$ retinoid-related orphan receptor γt ($RoR\gamma t)^+$), pDCs ($Lineage^-HLA\text{-}DR^+CD123^+$), mDCs ($Lineage^-HLA\text{-}DR^+CD11c^+$), and their activation status were carried out by flow cytometry. Additionally, the presence of these cells were determined by immunohistochemical staining.
Results: The proportions of circulating pDCs, mDCs and $CCR6^+$Tregs were decreased whereas Th17 cells were increased in *H. pylori*-infected children.The expression of costimulatory molecules such as CD83 and CD86 on surface of DCs were unchanged as compared with control group.
Conclusion: We showed that circulating DCs and $CCR6^+$Tregs probably accumulate in the child *H. pylori*-infected gastric mucosa.

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Cell mediated cytotoxicity in 'untouched' whole blood

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Introduction: Cell-mediated cytotoxicity is a major effector pathway of the immune system for protection against pathogens and tumours. Cellular cytotoxicity is mediated by various cell types such as natural killer (NK) cells and cytotoxic T lymphocytes (CTL) and it involves several mechanisms for the induction of death in the target cells. Different standard methods to study cell-mediated cytotoxicity are problematic, as they rely on radioactivity, and have variable efficacy of target cell labelling, with long tedious protocols. We have developed a novel methodology to study cytotoxicity that is more sensitive, faster, more accurate, more reproducible, and less harmful than current methods.
Methods: Cytotoxic activity was measured as the ability of cytotoxic cells to lyse K562 target cells previously loaded with Calcein-AM vital stain. After spiking a known number of fluorescent viable K562 target cells into whole blood, cell mixtures were incubated for 2 hours in a cell incubator and the remaining spiked cells were counted by flow cytometry. In order to discriminate nucleated cells, erythrocytes, and debris, unlysed blood was vitally stained with a cell permeable DNA fluorescent dye. Cell-mediated lysis was measured by comparing target counts for different target-effector ratios.
Results: K562 cells presented a uniform staining pattern with Calcein-AM and EGFP-K562 cells but presented a progressive silencing of the protein which required repeated isolation of high-purity single cell clones by limiting dilution or by cell sorting. Cell counting of target and effector cells was assessed by performing serial blood

dilutions with a fixed number of K562 cells and the novel methodology presented good linearity and reproducibility. Cytotoxic activity is measured as the ability of cytotoxic cells from unlysed whole blood to effectively lyse K562 cells at a four different effector-to-target (E/T) cell ratios (1:3, 1:1, 5:3 and 10:3). Our results showed a decrease in the number of K562 cells compared to controls.
Conclusion: We have developed a novel and simple methodology to measure cytotoxic activity. Our results show that it is fast, accurate and reproducible. Moreover, it preserves the cellular function with minimum sample manipulation. Since the cytotoxicity of the dyes used is relatively low, this method can be broadly applied to studies of innate immune response to tumors and infections, especially where target-killing activity might be compromised by small volume samples or low frequency of cytotoxic cells.

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No-lyse no-wash new strategy for CD34+ for absolute cell counting without beads
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Background: Flow cytometry has classically been used to detect CD34+ cells. First assays consisted of using indirect immunofluorescence techniques, red-cell lysing procedures, and centrifugation and washing steps. CD34+ counts were obtained using a dual-platform technique, also known as the Milan Protocol. Since then, different methodologies have been described for improved detection of CD34+ cells (ISHAGE method).
Methods: Red blood cells contain hemoglobin, a molecule that readily absorbs violet laser (405 nm) light, whereas leukocytes and platelets/debris do not, resulting in a unique scatter pattern when observing human whole blood in the context of blue (488 nm) and violet (405 nm) side scatter (SSC). Inclusion of the Attune NxT No-Wash No-Lyse Filter Kit in the Attune NxT Flow Cytometer filter configuration allows simultaneous measurement of both blue and violet side scatter and the differentiation of red blood cells and leukocytes based on light-scattering properties alone in dilute samples. Leukocytes are outnumbered by red blood cells ~700-fold in whole blood. At lower sample dilutions required for enumeration of rare cells, significant coincidence of the target cells with red blood cells forces the use a fluorescence threshold and makes this differentiation difficult. Violet side scatter remains useful however, as the parameter height remains relatively constant even in the presence of coincident red cells. Unlysed blood samples were simultaneously stained with PE-CD34 and FITC-CD45 and a CD45 fluorescence threshold was set to eliminate red cells.
Results: The CD45-stained cells appeared on the bivariate plots as a region of dots clustered in three main populations, consisting of lymphocytes, monocytes and polymorphonuclear cells. CD34+ were easily identified as CD45dim cells. Resolution of leukocytes from red blood cells in whole blood is improved by incorporating violet 405 nm side scatter. Volumetric syringe pumps enabled absolute cell counting without beads and color compensation was not needed when PE-CD34 was excited using the yellow laser.
Conclusion: The rapid sample collection rates and inclusion of the Attune NxT No-Wash No-Lyse Filter Kit on the Attune NxT Flow Cytometer allow identification of leukocytes by scatter properties alone in dilute samples and allows better scatter resolution for more concentrated samples. This no-wash, no-lyse strategy can be used in combination with fluorescent probes that are specific for markers expressed by leukocytes as well as to identify CD34+ progenitor cells. Applications of this technology can be used both in basic research and clinical research laboratories.

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Standardized compensation setup for the ClearLLab LS* (Lymphoid Screen) application
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Introduction: The ClearLLab Compensation kit* was developed as an accessory to the ClearLLab LS (Lymphoid Screen Panel)*. Standardization is ensured by the provision of a comprehensive setup that includes reagent, hardware, sample preparation and analysis methods. Flow-Set PRO Fluorospheres target values are provided along with the ClearLLab LS application for setting compensation with the 10-color Compensation kit using auto-setup scheduler on the Navios Flow Cytometer.
Study: Prepared specimen age for 5 whole blood and 3 CYTO-COMP control cells samples was tested up to 26 hours at 2-8°C. Repeatability was tested on 4 blood and 1 control cell in 5 replicates. Within Laboratory Precision was tested on 3 Lots of kit by 3 operators on 3 instruments over 3 days. The % CV of the MFI and % spillover were monitored in this study and the ClearLLab LS was used as the Verify tube. Compensation setup over 3 weeks and

three instruments was tested by assessing the % gated population of the ClearLLab LS reagent. The panel was stored at 40°C for 2 months to further assess performance under stress. To aid in multiple Auto-Setup procedures with a single compensation kit, a minimum of 5 acquisitions per compensation kit at 10,000 cells per run was tested on normal whole blood and CYTO-COMP cells.

Results: The Flow-Set PRO Target values positioned negative populations in the first decade of the logarithmic scale on the Navios cytometer demonstrated on blood (80), bone marrow (58) and lymph node (3) samples. The intensity of each fluorescent dye conjugate was maintained when prepared cells are stored up to 24 hours at 2-8°C. The % CV for MFI and % spillover is ≤ 10% for Within-Run Precision and ≤ 20% for within Laboratory precision. For compensation setup over time and stress, the mean difference in marker % gated across time-points was less than 5%. The CV% of 5 acquisitions of single stained compensation kit was < 2% on all markers.

Conclusions: This study demonstrates the robustness and utility of the ClearLLab Compensation Kit by decreasing multiple compensation setup requirements and standardizing the workflow in a laboratory setting for 10 color flow cytometry applications.

** ClearLLab LS & ClearLLab Compensation Kit is not yet available for in vitro diagnostic use.*

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Performance Testing of the ClearLLab LS (Lymphoid Screen)*

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Introduction: Use of single color CE-IVD antibody-fluorochrome conjugates & Laboratory Developed Tests (LDTs) for identification of hematolymphoid populations entails extensive sample preparation, verification and validation, thus increasing workflow time and complexity in inventory management. To simplify the process and to enable utility of a standardized panel, the ClearLLab LS*, a 10-color, 12-antibody dried lymphoid screening panel was designed for use on the Navios Flow Cytometer. Its performance was assessed on fresh and aged specimens collected in different anticoagulants. The repeatability, reproducibility and the limits of detection of the ClearLLab LS panel were also evaluated.

Method: Specimen age was evaluated for 20 blood specimens up to 26 hours for K_2EDTA and K_3EDTA and 48 hours for ACD and Heparin anticoagulants at RT (18-25°C). The performance of the processed specimens were evaluated, for storage up to 5 hours at RT (18-25°C) and 26 hours at 2-8°C. The repeatability of the panel was determined on a total of 9 specimens (3 specimens each of blood, bone marrow and lymph node), with10 replicates each. The reproducibility of the panel was evaluated by 3 operators over 5 days, at 2 time-points a day, 2 repeats per time-point on 2 Navios Flow Cytometers using control cells The detection limits was calculated for the 72 Blank and 120 Low level Samples.

Results: The % mean difference and 95% Confidence Interval (CI) of the % gated positive cells for each marker across all anticoagulants was within 5%. The drift over specimen age and prepared specimens was calculated for all anticoagulants. The drift and the 95% CI for B, T and NK lineage was within 5% of % positive gated populations. The repeatability and reproducibility of markers with greater than 20% of CD45+ positive population was under 5% and10% respectively and markers with less than 20% CD45+ positive population was under 10% and 15% respectively. The LOD for all markers in the panel was less than 1%.

Conclusion: Similar performance of biological specimens across anticoagulants and age as well as low Coefficient of Variation (CV) on clinical and normal specimens demonstrates the potential for adoption of panels such as the ClearLLab LS reagent panel across geographies for improved standardization.

**ClearLLab LS is not yet available for in vitro diagnostic use.*

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Application of human single color IFN-gamma ELISPOT assay to detect gluten-specific T-cell responses in healthy subjects

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Introduction: Coeliac disease (CD) affects about 1% of the population in many regions of the world. This figure may be even larger, as it is believed that about 85% of those affected are currently undiagnosed due to variable signs and symptoms[1]. Gluten-specific HLA-DQ2 and DQ8 restricted CD4 T cells are central to the pathogenesis of CD. Gluten-specific T cells are present in peripheral blood due to antigen driven expansion in the intestine and subsequent spillover via the systemic circulation. Such T cells are measurable by overnight culture of peripheral blood mononuclear cells (PBMC) stimulated with gluten antigens and single cell cytokine secretion in an ELISPOT assay. Therefore, the objective of this study is to evaluate the performance the human single color IFN-gamma ELISPOT assay using antigenic peptides from gliadin, the immunogenic component of gluten, in order to predict subjects potentially intolerant to gluten and asymptomatic CD.
Methods: As stimulus for IFN-gamma secretion by HLA-DQ2 restricted CD4 T cells, a pool of 4 A-gliadin peptides was used[2]: p57–73 (QLQPFPQPQLPYPQPQS), p57–73 QE65 (deamidation product), AG01E9 p77-93 QE85 (QLQPFPQPELPYPQPQP) and AG02E9 p77-93 QE85 (QLQPFPQPELPYPQPQL). Fifteen samples of PBMC from healthy donors were tested by IFN-gamma-detecting ELISPOT assay ($4x10^5$ cells/ well). The aim of the experiment was to find positive responders to the coeliac peptide pool (50µg/ml). Subsequently, using the positive responders samples, C-pool antigens were titrated to establish the optimal concentration for the ELISPOT assay.
Results: Upon stimulation with the C-peptide pool, cells from 5 samples among the 15 tested samples responded with the spot numbers ranging from 18±3 to 217±24. The titration using those 5 samples showed that 4 samples responded with the spot numbers ranging from 39±7 (25µg/ml) to 297±65 (100µg/ml). The highest spot numbers were seen at a concentration of 100µg/ml.
Conclusions: We could identify 4 samples from healthy individuals, HLA-DQ2 restricted, which responded to the gliadin-derived antigens, suggesting that such patients could present an undiagnosed intolerance to gluten. Moreover, we have been assessing IFN-gamma ELISPOT assay as a useful technique that allows relevant T cells to be analyzed and quantified in peripheral blood from individuals with potential and undiagnosed gluten intolerance. Additional experiments evaluating the assay performance, precision and specificity will be performed.
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CD123 expression on acute leukemia blasts, hematogones, and monocytes; used as Minimal Residual Disease Marker

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Introduction: In recent studies, CD123 has been described as over-expressed on leukemia progenitor cells and low or absent on normal hematopoietic stem cells suggesting that IL-3Rα could represent an important target for the development of new anti-leukemic drugs and a minimal residual disease (MRD) marker. In the present study, we retrospectively analyzed the CD123 expression on blast cells in bone marrow or blood samples from patients with AML, ALL or RAEB and the disease evolution of these patients. CD123 was also measured on B-precursors and on monocytes.
Methods: From 2011 to 2016, 98 patients suffering from hematologic malignancies including 70 AMLs, 21 ALLs (18 B-ALLs and 3 T-ALLs) and 7 RAEBs were retrospectively analyzed in regard to CD123 (clone-FITC 763, BD Pharmingen) expression measured by flow cytometry (FCM) on myeloid and lymphoid blasts. CD123 was also analyzed on patients with hematogones (n=23) and on monocytes from 26 AML patients. FCM was performed on 5 and 10-colour flow cytometers with panels to precise leukemia-associated aberrant immunophenotype.
Results: Among the 98 patients 57 had a CD123 over-expressed. 47 patients suffered from AML. Among these 47 patients, 5 were lost in follow-up and 25 were in refractory/relapse phase or dead. 7 patients, including 6 B-ALL and 1 T-ALL, had a CD123 over-expressed. Among these 7 patients, 5 were in refractory/relapse phase or dead. Among the 7 RAEBs, 3 patients had a CD123 over-expressed and are still alive. The mean value +/- SD of the

%CD123 on CD45low in all and in the CD123 positive cases of AML, ALL and RAEB was respectively 36 +/- 29.7% and 51 +/- 24.7%, 18 +/- 28.2% and 52 +/- 24.5%, 30 +/- 30.7% and 60 +/- 20.5%. CD123 over-expression was not observed on B-precursors but a positivity was observed on monocytes in 33 of 158 samples from 26 AML patients with a total mean value +/- SD of the % CD123 of 15 +/- 20.4%. Among the 44 patients undergoing long-term follow-up, 10 shifts and 4 inversed shifts were observed in the CD123 expression.
Conclusions: Our observations confirm that CD123 is very frequently expressed on leukemia blast cells, in about 2/3 of AMLs and probably in 1/3 of ALLs with a rare loss of expression with antigenic shift of the disease. While CD123 is not expressed on hematogones, it is in monocytes, thus interfering with easy use of CD123 as only MRD marker.

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Improving inter-instrument standardization in flow cytometry
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Introduction: Recent focus of the pharmaceutical industry in immuno-oncology, auto-immunity, infection diseases and cell therapies, has led to an increase in the requirement of flow cytometry in support of all the drug development phases from fundamental research to late clinical development stages. Conducting flow cytometry in the context of longitudinal, and most-often, multi-center, clinical trials in a regulated environment requires robust instrument-to-instrument standardization process in order to generate high-quality data from multiple sites and instruments.
Methods: Using a process described by Becton Dickinson where hard dyed beads (CS&T) are used for the daily application settings, 19 BD FASCSCanto® II (registered to Becton, Dickinson and Company) flow cytometers located in Indianapolis, Geneva and Singapore were cross-standardized. However, variability between instruments was greater than expected. Thus a new approach in which specific fluorochrome bound beads (BD FC beads) are used for the application settings is currently evaluated.
Results: Preliminary data comparing the two approaches will be presented.
Conclusions: Benefits of this instrument-to-instrument standardization include streamlining of the daily work flow process, cost efficiencies and highly comparable data for intra and inter-patient analyses enrolled in global clinical trials.

72 (Selected Best Poster Abstract Presentation)

Interferon-gamma production based virus specific T cell detection by functional flow cytometry test after allogeneic haematopoetic stem cell transplantation
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Introduction: After allogeneic haematopoietic stem cell transplantation (HSCT) EBV, CMV or adenovirus reactivation can be fatal. Besides antiviral therapy adoptive transfer of virus specific T cells selected by Prodigy technology is an increasingly available and rapid treatment option for these patients.
Methods: In our institution 13 patients (9 children, 4 adults) have recieved virus specific T cell therapy from third party donors since May 2015: 9 CMV, 2 EBV, 3 adenovirus, and 1 BK virus infected patients were treated (some of the patients recieved treatment for multiple viruses). Donors were selected during screening procedure by functional flow cytometry test based on interferon-gamma (IFNγ) production of T cells in the presence of viral antigens (Peptivator). Graft purity after Prodigy magnetic selection was also determined with a similar flow cytometry based technique. To evaluate the clinical efficacy of the treatment viral copy numbers measured by PCR methodology were monitored after T cell therapy.
Results: Results of 149 healthy donors and 221 screening tests were evaluated regarding the frequency of virus specific CD4+ and CD8+ T cells (99 CMV, 40 EBV and 40 adeno virus and 42 BK virus tests). Average percentage of virus specific T cells in healthy donors were as follows within the CD4+ and CD8+ T cell populations, respectively: 0,1415 % and 0,2923% for CMV; 0,0074% and 0,0718% for EBV; 0,033% and 0,016% for adenovirus. At 0,01% cut off value, 80% of the screened persons were suitable as donors for CMV, and 50% for EBV and adenovirus antiviral treatment. The average purity of the grafts was 94% for CMV, 84% for EBV and 65% for adenovirus T cell products. Although the average recovery was 30%, the mean number of IFNγ producing T cells was highly sufficient, resulting

in8,76·10^5 of CD4+ T cells and 1,5·10^{6}of CD8+ T cells for immunotherapy. Viral copy numbers decreased in most cases few weeks after T cell therapy with the resolution of clinical symptoms. GvHD was not observed.
Conclusion: Administration of virus specific T cells from third party donors selected with Prodigy technology is an effective antiviral treatment option after allogeneic HSCT. Functional flow cytometry test is a quick and sensitive method for donor screening and purity check of the T cell grafts, as well as for monitoring immunoreconstitution of virus specific T cells after T cell therapy.

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Monocytes and U937 cells are differently responsive to bacteria lysates from 3 different Campylobacter jejuni strains

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Introduction: The health burden of *Campylobacter jejuni*-associated pathologies are all factors driving current impetus for gaining further insight into *C. jejuni*-mediated disease pathogenesis. Both innate and acquired immunity are thought to play critical role in host resistance to infection and pathogenesis of campylobacteriosis. Among the *C. jejuni* virulence factors, the cytolethal distending toxin (CDT) is a well characterized bacterial genotoxin, contained in outer membrane vesicles (OMVs). In this work, we focus on the cellular effects of *C. jejuni* ATCC 33291 and *C. jejuni* ISS 1 strains cell lysates (CCLys), positives for the *cdt*A, *cdt*B, *cdt*C genes, compared to *C. jejuni* 11168H *cdt*A mutant, in both U937 cell line and donor monocytes.
Methods: *C. jejuni* cell lysates, containing OMV and CDT, of *C. jejuni* ATCC 33291 and *C. jejuni* ISS 1 were added to monocytes and U937 cells and after 12, 24, 48 and 72 hours, cells were analysed for mitochondrial, lysosomal and cell death features, p53 and Bcl-2 status, CD54, CD14, CD16 and CD59 alterations. Furthermore, specific phenotype markers were used.
Results: We report that CCLys induced mitochondrial alterations, apoptotic behaviour, increment of endolysosomal compartment and Endoplasmic Reticulum stress, in both cellular models. Moreover, CD54 and CD59 molecules were altered in both cell lines. CD54 (ICAM-1), an adhesion molecule present on the surface of monocytes and other antigen-presenting cells, contributes considerably to the capacity of these cells to adhere and to play an important role in bacteria (and bacterial toxin) internalization. Also for CD59 molecule a direct involvement in cell activation have been demonstrated. Our results highlighted a differential increase of CD59 and significant peaks and drops of surface CD54, during all time course investigated.
Conclusion: In the present work, we determined the functional alterations in monocyte/macrophage system, also distinguishing between the classical (CD14+/CD16"') and intermediate or inflammatory (CD14+/CD16+) phenotypes. These findings demonstrate that CCLys are able to induce lethal and sublethal (and reservoir-transforming) effects in U937 and monocytes, depending on the specific *C. jejuni* strains.

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Neutrophil antibody detection in sera of neutropenic pediatric patients by microbeads coated with human neutrophil antigens

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Introduction: Neutrophil antibodies (abs) with prevalent specificity against Human Neutrophil Antigens (HNA) are implicated in Autoimmune Neutropenia (AIN) and in Neonatal Alloimmune Neutropenia (NAIN). The Flow Cytometry-Granulocyte Immunofluorescence Test (flow-GIFT) and the Granulocyte Agglutination Test (GAT) are the current gold standard methods for the detection of HNA abs. However, flow-GIFT shows limited sensitivity, particularly so if not performed with a panel of typed neutrophils including homozygous HNA-1a and -1b typed donors, which show higher antigen density. Recently, a method based on microbeads coated with purified HNA antigens (LabScreen Multi, LSM, One Lambda) has been proposed for the detection of circulating HNA abs. In this study we compared the results of HNA ab screening obtained by LSM versus flow-GIFT in sera of suspected AIN pediatric patients and in positive control (PC) sera (CQ INSTAND).

Methods: We tested 59 suspected AIN cases, 2 suspected NAIN cases and 11 PC sera with specific HNA abs. Flow-GIFT was performed against a 4-cell panel including at least one homozygous HNA-1a and -1b healthy donors. FACSCanto II and FACSDiva software (BD Biosciences) were used for sample acquisition and analysis. LSM was performed according to the manufacturer's instructions. The cut-off values were set by testing a pool of 30 non-transfused male sera.
Results: Test time was shorter with LSM. Flow-GIFT was positive in 36 sera from suspected AIN (61%); LSM was positive in 21 of the latter (58%). Flow-GIFT detected 11 HNA-1a positive sera while LSM identified 5 of them; moreover, 1 of 8 HNA-1b sera positive by flow-GIFT was also positive by LSM. LSM detected 1 HNA-1a and 1 HNA-1b specificities not identified by flow-GIFT. Two (9%) of 23 sera negative by flow-GIFT were positive by LSM, both with HNA-1a specifity. Screening of NAIN sera by the two methods was concordant for HNA-1b specificity. Flow-GIFT identified all the specificities in 11 PC sera containing HNA-1a, HNA-1d, HNA-2, HNA-3a, HNA-3b, HNA-4a abs. LSM missed one HNA-3a positive serum and identified a second specificity for HNA-3b in two HNA-3a positive sera.
Conclusions: LSM correlated well with the gold standard methods for allo- and autoantibody detection. Specificities detected by two methods were not always the same, especially for HNA-3a. The use of beads coated with well defined antigen densities could overcome problems due to limited availability of cell panels from typed homozygous donors. Potential advantages and disadvantages using beads with purified antigen versus typed cells will be discuss.

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Endothelial Progenitor Cells in patients with diabetic peripheral neuropathy - preliminary results
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Introduction: Diabetes Mellitus (DM) is the most common metabolic disease worldwide affecting an ever-growing number of people and has a heavy impact on public health systems. Individuals with DM often develop complications of the vasculature leading to serious disabilities. Endothelial Progenitor Cells (EPCs) are involved in vascular homeostasis and repair. However, there are limited data on the role of EPCs in the development of diabetic peripheral neuropathy (PN), which is a common complication affecting almost 30% of subjects with DM. The aim of this study was to examine the association of EPCs in patients with type 2 DM
Methods: After venipuncture Peripheral Blood Mononuclear Cells (PBMCs) were obtained from 40 adult non-smoker donors without clinical macrovascular disease: 15 non-diabetic controls (♀/♂: 12/3, aged 56.6±9.5) and 25 patients with DM [19 without PN (no-PN) (♀/♂: 6/13, aged 61.3±7.4) and 6 with PN (♀/♂:1/5, aged 66.0±5.2)]. The cells were stained with monoclonal antibodies against CD45, CD34 and CD309 obtained from BD Biosciences. Acquisition and analysis of $1x10^6$ events per subject was performed by the six-color flowcytometer BD FACSCanto using the modified ISHAGE Protocol to define CD45dimCD34+CD309+ cells. Statistical analysis was performed with SPSS 17
Results: Our no-PN patients had: median disease duration 9.6±6.7 ys, retinopathy 2/19 (10.5%), hypertension 12/19 (63.2%), dyslipidemia 14/19 (73.7%), body weight 91.8±17.7 kg, BMI 32.0±5.9 kg/m2, waist 108.7±11.6 cm. The PN patients had: median disease duration 16.5±6.8 ys, retinopathy 4/6 (66.7%), hypertension 6/6 (100%), dyslipidemia 4/19 (66.7%), body weight 95.0±17.8 kg, BMI 31.9±5.5 kg/m2, waist 109.3±12.4 cm. Characteristics of the control group: 2/15 (13.3%) presented hypertension, 4/15 (26.7%) dyslipidemia, body weight 73.1±12.9 kg, BMI 25.9±3.8 kg/m2, waist 97.5±11.5 cm. EPCs median was found in the no-PN patients 62 (range 31-89), in PN 79 (range 57-121) and in controls 40 (range 26-69). Statistical analysis of EPCs median using the Mann–Whitney U test gives p values as follows: for PN vs. controls p=0.032, for no-PN vs. controls P=0.118, for no-PN vs. PN p=0.239. Evaluation of the median of EPCs of all groups by the Kruskal Wallis test gives a trend for significance (p=0.067)
Conclusions: The number of EPCs was significantly higher in patients with PN in comparison with controls. This finding, although preliminary, may imply that there is either an effort for restoration of the damaged peripheral nerves or a homing deficiency. More research is warranted to clarify the role of EPCs in diabetic PN

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An early polyfunctional CMV-specific memory T cells recovery is associated with a better control of CMV reactivation in kidney transplantation

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Introduction: Polyfunctional T cell responses are associated with lower viral loads in chronic infections. In renal transplantation, Cytomegalovirus (CMV) remains one of most frequent and deleterious viral infections. Characterizing CMV-specific polyfunctional memory T cells could allow evaluating the individual infectious risk, and providing a tool to guide the management of preemptive or prophylactic antiviral treatment in renal transplant recipients.

Methods: We prospectively compared CMV-specific-memory T cells of 20 healthy donors and 22 renal transplant patients on the day of transplantation, 6 weeks, 3 and 6 months after transplantation. All participants were recruited at University Hospital of Nancy (France) and gave an informed consent. Fresh PBMC were stimulated overnight with medium or overlapping peptides from IE-1 or pp65 viral proteins in the presence of brefeldin-A and co-stimulatory antibodies (CD28 and CD49d). Intracellular cytokines (IFN-gamma, TNF-alpha, IL-2) production and phenotype profiles (CD3, CD4, CD8, CD197, CD45RA, CD95, amine-reactive dye) of CMV-specific T cells were evaluated by multiparametric flow cytometry. An increase of at least 2-fold percentage of cytokine secreting T cells after IE-1 or pp65 stimulation compared to medium culture only identified CMV-specific immune responses. Concomitant cytokine expression and differentiation phenotype of CMV-specific CD4 were evaluated. The newly described polyfunctionality index was used to compare CMV-specific T cells between CMV-seropositive patients with or without CMV-reactivation.
Results: Among the 15 CMV-seropositive patients, 7 had a symptomatic CMV reactivation. The numbers of CMV-specific CD4 or CD8 T cells or their differentiation profile at the day of transplantation was not different between patients with or without CMV-reactivation post-transplantation. Six weeks after transplantation, the polyfunctionality index of IE-1-specific CD4 T cells in patients who had a CMV-reactivation was lower ($p<0.05$) compared to those without CMV-reactivation ($p<0.05$). There were no significant differences in polyfunctionality index at 3 and 6 months after transplantation. However, bi and tri-functional IE-1-specific CD8 T cells were over-represented in patients without CMV-reactivation ($p<0.05$ compared to patients with CMV-reactivation). Patients with CMV-reactivation had higher proportions of effector-memory cells among IE-1-specific CD8 T cells 3 months after transplantation compared to patients without CMV-reactivation ($p<0.005$).
Conclusions: Our results suggest that IE-1-specific, polyfunctional and less differentiated CD4 and CD8 T cells are associated with a decreased risk of CMV-reactivation. Less terminal lymphocyte differentiation associated with a superior polyfunctionality index could be used as predictive biomarkers of viral replication control in the renal transplant setting. Further studies are warranted to confirm these data.

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Establishing risk factors for the development acute GvHD after allogeneic hematopoietic stem cell transplantation using 13-colour flow cytometry panels

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Introduction: The development of Graft versus Host Disease (GvHD) is a multistep process including priming of the immune response by the systemic inflammation induced by the conditioning, alloreactive T and B cell activation, costimulation, expansion and differentiation and trafficking of alloreactive T cells finally resulting in the destruction of target tissue by effector T cells. We designed six multi-colour flow cytometry panels in order to establish risk factors for the development of GvHD.
Method: Peripheral blood mononuclear cells (PBMCs) of healthy donors were used to validate and set up the multi-colour flow cytometry panels. Cell subtypes and markers of interest were divided over six panels using a maximum of 13 different fluorochromes. Compensation matrices were set up using compensation beads for each of the panels. Alignment and rainbowbeads were used to calibrate the flow cytometer (LSRFortessa) before each measurement to ensure stable and comparable measurements that can later be used for unbiased computational biomarker discovery.

Results: We designed a panel containing T cell markers identifying their memory, activation and exhaustion status; a panel containing B cell markers identifying their memory, IgG subclass and activation status; a panel identifying four different dendritic cell subtypes, classical and non-classical monocytes and NK cells; a panel identifying four types of innate lymphoid cells; a panel identifying T cells by their transcription factor expression; a panel identifying Th17 like cells and NKT cells. For each panel we calculated the minimal number of cells that need to be analysed to be able to identify the subtype with the lowest expected frequency.
Conclusion: Six multi-colour flow cytometry panels were successfully set up and validated on healthy donor peripheral blood. By calibrating before each patient sample measurement we aim to safeguard the stability of the flow cytometer to obtain robust data for unbiased computational analyses.

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For a reasonable and realistic flow cytometry Method Validation toward iso 15189 accreditation
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Clinical lab must have to process toward ISO 15189 accreditation but Clinical flow cytometry (FCM) faces difficulties for that, mainly due to the sample fragility and diversity, rarity of some phenotypes and the large number of Analyses. For the same reason, internal (IQC) and external (EQC) quality controls are rarely available. Analyses are complex including multiple results per analysis and/or multiple analyses for one result (panels) and frequently need adaptation to clinical and biological requirements. Methods and data analysis are in continuous progress and last but not least are costly. But FCM can benefit from accreditation.
Here we propose a reasonable and realistic solution for the method validation in the process of accreditation under flexible scope as a result of several meetings of experts in Clinical Cytometry:
Method validation: precision: **9 to 15 repeats**, on 2 levels and on one representative type of sample (blood, Bone Marrow). Reproducibility: daily repeat of IQC when available (rarely), one type of sample, two levels. Accuracy: EQC, one type of sample, 2 levels, 2 – 4 times per year. Global uncertainty can include inter-laboratory IQC comparisons. Contamination risk by 3-5 repeats of 3 high / 3 low value samples. Working range, Linearity, Limit of quantitation on one cell population, one type of sample, one analysis. Method comparison between (mirror) instruments or change of technique on 30 to 35 representative samples on different levels over the working range.
Accreditation report: in order to facilitate updating, any repeat must be avoided. Information must be reported at 4 different levels on: one FCM generic document for all common information (material, environment method) and part of method validation (repeatability, reproducibility, LOQ, contamination). Several FCM specific documents, one per panel (multiple results) that details specificities (panel composition, sample preparation, specific risks). Standard Operating Procedures (SOP) detail all technical information with need for frequent updating (reagent specification, dilution, gating strategy, typical results, alerts). Finally: technique information, reference data, automatic comments are listed in the Lab Informatics Management System to be edited with results for interpretation.
In conclusions: These recommandations should help lab to enter an efficient process of a global accreditation of the cytometry platform for quality assurance and the benefit of patients.

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A new Primary Immunodeficiencies Screening panel in ready to use dry tube
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Primary Immunodeficiencies are rare and difficult to detect except for severe common immunodeficiency syndromes (SCID). Lots of patients present episodes or recurrent infections of unknown origin. Most frequent genetic disorders concern complement or phagocytosis. Few have cell immunity dysfunction. More and more mutations are described that explain default in cell function and/or maturation but each entity is usually rare.

Routine lab tests are not sufficient for detection candidates to be further explored in details. **The aim** of this project is to set up a screening tube, compatible with different systems, possibly standardized and easy to use at low frequency in the middle of all routine tests.
Methods: we designed a 8 color, 11 parameter panel with absolute cell count, to be analyzed on any instrument with at least 8 colors detectors, in a single step with long life reagents (Duraclone Beckman-Coulter), no lyse no wash sample preparation.
Results: Our results show that the panel is easy to set up on FACS CANTO II (BD Biosceinces); NAVIOS and CYTOFLEX (Beckman-Coulter) instruments, using lysis systems routinely used in the labs. The main immune cell populations can be counted : CD4+ or CD8+ T, B and NK cells, Monocytes, Granulocytes, Eosinophiles. Naïve and mature T (CD45RO/CD27), B (CD38/CD27/IgD) and NK (CD16High CD56low) cells can be quantified as well as activated (CD38+) T, NK cells. Instrument settings can be standardized based on targets for fluorescence intensity provided for different systems. A typical template is available. Drop-in parameters can be added in systems with more photodetectors.
In conclusion: such a panel, can be easily implemented in basic immunology lab for detection of potential immunodeficiency to be further explored with orientated panel, functional tests and ultimately orientated genotyping. The panel can also be used for secondary immunodeficiencies as observed in chronic inflammatory diseases or sepsis.