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Programme

ESCCA COURSE PROGRAMME

Lectures for Basic Leukemia, Cytometry Toolbox 1 and Cytometry Toolbox 2

Jaco Kraan *(Rotterdam, NL)*	Instrument set-up, calibration and measurement of performance
Claudio Ortolani *(Venice, IT)*	Multicolor compensation
Bruce Greig *(Nashville, US)*	Education and training requirements for performing clinical flow cytometry and how to prepare for an inspection?
Maurice R. G. O' Goorman *(Chicago, US)*	Primary immunodeficiencies
Bruno Brando *(Milan, IT)*	Absolute cell counts – positive/negative analysis
Elizabeth Stone *(Nashville, US)*	Sample processing" and intracellular staining
David Barnett *(Sheffield, UK)*	EQA for immune monitoring
Judith Chezar (*Nahariya,IL)*	"Evaluation of spherocytosis by flow cytometry" and cell cycle analysis

Basic Leukemia
Case studies by Elizabeth Stone, Borowitz, Judith Chezar

Cytometry Toolbox 1
Wet lab by Claudio Ortolani, Bruno Brando, David Barnett

Cytometry Toolbox 2
Wet lab by Jaco Kraan, Bruce Greig, Maurice R.G. O' Goorman

Lectures for Advanced Leukemia, Lymphoma and Myelodysplasia

Alberto Orfao *(Salamanca, ES)*	Normal maturation of granulocytic cells and normal maturation of other myeloid lineages
Julia Almeida *(Salamanca, ES)*	Normal maturation of monocytic cells
Raul Braylan *(Gainesville, US)*	Normal and reactive lymph nodes
Sergio Matarraz *(Salamanca, ES)*	Immunophenotype of CD34+ cells in myelodysplasia
David Barnett *(Sheffield, UK)*	EQA for leukemia
Brent Wood *(Seattle, US)*	MRD in AML and diagnosis of Hodgkin's Lymphoma by flow cytometry
Margarida Lima *(Porto, PT)*	T-cell leukemia/lymphoma
Maryalice Stetler-Stevenson *(Bethesda,US))*	B cell chronic lymphoproliferative disorders

Stephen Richards *(Leeds, UK)* PNH by flow cytometry

Jeannine Holden (*Atlanta,US)* MRD in ALL

Advanced Leukemia
Case studies by Wood, Richards, Holden

Lymphoma
Case studies by Braylan, Lima, Stetler-Stevenson

Myelodysplasia
Case studies by Orfao, Almeida, Matarraz

19:00 – 24:00
Dinner I: boat trip from Martinianleger "OCEANA"

JOINT MEETING OVERVIEW

	Tuesday Sept. 16th	**Wednesday** Sept. 17th	**Thursday** Sept. 18th	**Friday** Sept. 19th	**Saturday** Sept. 20th
Morning	ESCCA Course	ESCCA Course / DGfZ Program	Joint Plenary Session (I)	ESCCA Program	ESCCA Program
Noon	ESCCA Course	ESCCA Course / DGfZ General Assembly	Joint Plenary Session (II) / Joint Poster Session / Industrial Exhibition	ESCCA Program	ESCCA Program
Break					
Afternoon	ESCCA Course	ESCCA Course / DGfZ Program	Joint Poster Session / Industrial Exhibition / Joint Plenary Session (III)	ESCCA General assembly	
Late Afternoon	ESCCA Course / DGfZ Scientific Opening Session	ESCCA Course / DGfZ Program	Joint Session Scientific Industrial Presentations / Keynote Lecture	ESCCA Program	
Evening	Get-Together Opening Industrial Exhib. (DGfZ Conference & ESCCA Course)	Gala Dinner (DGfZ Conference & ESCCA Course)		Gala Dinner (ESCCA Conference)	

DGFZ MEETING PROGRAMME

DGfZ Meeting Programme

Tuesday, Sept 16

16:00 – 17:00	**Registration**

17:00 – 18:45
Scientific Opening Session: Epidemiological diseases with tremendous impact in Africa
Chairs: **Susan Müller** (Leipzig, DE), **Gero Brockhoff** (Regensburg, DE)

Wolfgang Göhde (Münster, DE)	Adapted technologies new concepts for diagnosis of three major diseases in Africa: HIV-AIDS, Malaria, and Tuberculosis
Yves Traoré (Bobo-Dioulasso, BF)	HIV/AIDS, Malaria, and Tuberculosis: Three killer diseases in Africa; is their any hope for African countries?

Get together – Opening Industrial Exhibition

Wednesday, Sept 17

08:00 – 16:30	**Registration**

08:30 – 10:15
Session I: Cytometry in Microbiology and Biotechnology
Chairs: **Susan Müller** (Leipzig, DE), **Frank Stahl** (Hannover, DE)

Michael Blaut (Nuthetal, DE)	Application of flow cytometry to the analysis of the gut microbial ecosystem
Günther (Leipzig, DE) et al.	A new fluorescent in vivo detection method for polyphosphate accumulating bacteria
Peterbauer (Linz, A) et al.	Impact of culture conditions on the surface marker profile and other properties of stem cells from amniotic membrane and adipose tissue
Moretti (Hannover, DE) et al.	Characterization of mesenchymal stem cells isolated from human umbilical cord
Herber (Bergisch-Gladbach, DE) et al.	VioBlue a novel violet-excited dye for multicolor flow cytometry
Beck (Palo Alto, USA) et al.	Label-free flow cytometry on a microfluidic chip based on native fluorescence
10:15 – 10:45	Break (coffee)

10:45 – 12:30
KG-Prize awarding / DGfZ member's meeting

13:30 – 15:15
Session II: Tissue Analysis and Imaging
Chairs: **Johannes Wessels** (Göttingen, DE), **Josef Neumüller** (Wien, AT)

Paul Verkade (Bristol, UK)	Studying Intracellular transport using correlative light electron microscopy
Neumüller J. (Wien, A) et al.	Endothelial progenitor derived cells express the SR-B1 scavenger receptor and take up high density lipoprotein as ligand (A study using correlative microscopy, DAB photoconversion and electron tomography)
Pierzchalski A. (Leipzig, DE) et al.	Concentration measurement of fluorescent solutions by Slide Based Cytometry
Bader B. M. (Rostock, DE) et al.	Signal protein translocation in differentiating human neural progenitor cells: Generation of quantitative data for a systems biology approach from confocal 3D image stacks
Malkusch W. (Hallbergmoos, DE)	Cell based cytometry using wide field microscopy
Tarnok A.(Leipzig, DE) et al.	Cellular and humoral immune response to open heart surgery in children is modulated by methylprednisolone

16:00 – 18:00
Session III: Cancer Biology of Solid Tumors
Chairs: **Gero Brockhoff** (Regensburg, DE), **Simone Diermeier-Daucher** (Regensburg, DE)

Gyorgy Vereb (Debrecen, HU),	Role of EGF receptor tyrosine kinases in breast cancer. A biophysical approach
Diermeier-Daucher S. (Regensburg, DE) et al.	Initial mechanisms triggering ErbB mediated signaling - implications for efficient targeted therapy
Sassen A. (Regensburg, DE) et al.	Three-level based analysis of c-erbB receptor tyrosine kinases in breast cancer
Pachmann K. (Jena, DE) et al.	Influence of anti-her2/neu therapy on gene analysis of circulating epithelial tumor cells (CETC) under the influence of anti-her2/neu therapy
Kopinski P. (Bydgoszcz, PL) et al.	Inhibition of insulin-like growth factor type I receptor 1 (IGFR1) gene, but not epidermal growth factor receptor (EGFR) neither Her-2/neu orphan receptor genes, induced phenotype changes relevant to increased tumor susceptibility to immune system in A549 human lung cancer cells
Robert Murphy (Pittsburgh, USA)	Automated analysis of subcellular patterns in human protein atlas images: Application to identification of potential biomarkers for prostate cancer

19:00 – 24:00
Dinner I: boat trip from Martinianleger “OCEANA”

JOINT MEETING PROGRAMME

Joint Meeting Programme

Thursday, Sept 18

08.00 - 16.30	**Registration**
09.00 - 10.30	**Joint Plenary Session I** Cell Signaling chair: **Gero Brockhoff**, Regensburg, DE
	Yosef Yarden (Rehovot, IL) The EGF receptor family - spearheading a merger of signaling and therapeutics
	Fridtjof Lund-Johansen (Oslo, NO) High-throughput analysis of protein phosphorylation and protein interactions
	Joanne Mountford (Glasgow, UK) Functional characterization of cancer stem cells in CML
10.30 - 11.00	Break (coffee) – Joint Poster Session / Industrial Exhibition
11.00 - 12.30	**Joint Plenary Session II** Rare Event Analysis and Sorting Chair**: Wolfgang Beisker** (Neuherberg, DE)
	Patrizia Paterlini-Brechot (Paris, FR) Analysis of rare circulating cells in non-invasive diagnosis
	Leon Terstappen (Huntingdon Valley, US) FCM of circulating tumor and endothelial cells
	Christoph Klein (Regensburg, DE) Single-cell based genomic characterization of tumor cells
12:30 - 14.00	Lunch and **Poster Session** Chairs: Torsten Viergutz (Rostock, DE), Ulrich Sack (Leipzig, DE)
	Joint Session – Industrial Workshop
13.30 -14.00	**IQ Products / Trillium** – Improved Sepsis Diagnosis and Monitoring by Quantitative Flow Cytometry: Leuko64, a New Paradigm **Bruno Brando** (Milano, IT) **Marie-Christine Béné** (Nancy, FR)
14.00 - 15.30	**Joint Plenary Session III** Cell Function and Ageing Chair: **Gregor Rothe**, Bremen, DE
	Paolo Bianco (Rome, IT) Microvascular clonogenic postnatal precursors
	Gabriele Baerlocher (Bern, CH) Analysis of telomere length by FCM and FISH
	Paul Smith (Cardiff, UK) Loss of genomic integrity and escape from senescence

15.30 - 16.00	Break (coffee)
	Joint Session – Scientific Industrial Presentations (Chair: **Elmar Endl**, G**; Stefano Papa**, IT)
16.00 – 16.30	**Beckman Coulter –** New insights into the biology of chronic lymphocytic leukaemia - analysis of cell sorted sub-clonal populations **Chris Pepper** (Cardiff, UK)
16.30 – 17.00	**BD Biosciences** – Developments in the detection of intracellular antigens by flow cytometry **Andy Rawstron** (Leeds, UK)
17.00 – 17.30	**Partec GmbH** – High sensitivity microbiological applications performed on Partec flow cytometers **Danny Köhler**
17.30 – 18.30	**Keynote Lecture** **Andreas Radbruch** (Berlin, DE) Antibodies and B cell memory in immunity and immunopathology

ESCCA MEETING PROGRAMME

ESCCA Meeting Programme

Friday, Sept 19

08.00 - 16.30	**Registration**
09.00 - 10.30	**Plenary Session IV** Cell Therapy Chair: **Jan Gratama**, Rotterdam, NL
	Louis Casteilla (Toulouse, FR) Adipose tissue as a source of stem cells
	Graziella Pellegrini (Venice, IT) Epithelial tissue reconstitution using native or transformed stem cells
	Jolanda de Vries (Nijmegen, NL) Migration and effectiveness of tumor-antigen loaded dendritic cells in cancer patients
10.30 - 11.00	Break (coffee)
11.00 - 12.30	**Plenary Session V** Leukemia / Lymphoma Chair: **Mars Van't Veer**, Rotterdam, NL
	Gerrit Schuurhuis (Amsterdam, NL) Acute myeloid leukemia stem cell analysis
	Rajenda Damle (Manhasset New York, USA) The life and death of chronic lymphocytic leukemia B cells
	Jaco Kraan (Rotterdam, NL) Flow cytometric detection of leukemic cells in cerebrospinal fluid
12:30 - 14.00	Lunch

14.00 - 15.30	**Plenary Session VI** Platelet Analysis Chair: **Janos Kappelmayer**, Debrecen, HU
	Lorenzo Alberio (Bern, CH) Phenotypic and functional analysis of platelets
	Aaron Tomer (Beer Sheva, IL) Diagnostics of platelet autoimmunity
	Henk J. Broxterman (Amsterdam, NL) Platelet-induced angiogenesis and tumor growth
15.30 - 16.00	Break (coffee)
15.30 - 17.30	**Plenary Session VII** HIV / Vaccines / Microbiology Chair: **Mariam Klouche**, Bremen, DE
	George Janossy (London, UK) Cost-effective diagnosis of TB and HIV in resource-poor settings
	Laura Papagno (Paris, FR) T cell response in HIV infection
	Helen McShane (Oxford, UK) T cell response in different vaccination strategies against tuberculosis
	Short break (refreshments)
17.45 - 19.00	**GENERAL ASSEMBLY OF ESCCA**
20.00	Dinner II: Bremer Ratskeller, Am Markt

Saturday, Sept 20

08.30 - 12.00	**Registration**
09.00 - 10.30	**Plenary Session VIII** Myelodysplastic Syndromes Chair: **Maria Arroz**, Lisbon, PT
	Arjan van de Loosdrecht (Amsterdam, NL) New issues of MDS diagnosis and prognostication
	Lars Nilsson (Lund, SE) Gene profiling and phenotype of MDS stem cells
	Ghulam Mufti (London, UK) The involvement of the immune system in MDS: the role of FoxP3+ T reg cells
10.30 - 11.00	Break (coffee)
11.00 – 13.00	**Plenary Session IX** Interactive voting – controversies
Controversy A	Low abnormal cell numbers in chronic lymphoproliferative disorders Chair: **Ulrich Sack**, Leipzig, DE
	Andy Rawstron (Leeds, UK) They are really important!
	Mars van 't Veer (Rotterdam, NL) Who cares?
Controversy B	Pre-clinical assessment of myeloma by Flow Chair: **Bruno Brando**, Milan, IT
	Alberto Orfao (Salamanca, ES) Flow Cytometry provides clinically important information
	Amin Rahemtulla (London, UK) Flow has no role

Conference Organisation

4th European Clinical Cytometry Course & Joint Meeting of 8th Euroconference on Clinical Cell Analysis (ESCCA) & the 18th Annual Meeting of the German Society for Cytometry (DGfZ)

Conference chairs:
Gregor Rothe, Bremen (DE)
Gero Brockhoff, Regensburg (DE)

Organising Commitee:
Gregor Rothe, Bremen (DE)
Gero Brockhoff, Regensburg (DE)
Bruno Brando, Milan (IT)
Jan Willem Gratama, Rotterdam (NL)
Janos Kappelmayer, Debrecen (HU)
Mariam Klouche, Bremen (DE)
Claude Lambert, St. Etienne (FR)
Ulrich Sack, Leipzig (DE)

Course Chair:
Maria Arroz, Lisbon (PT)

Organising Committee:
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Mariam Klouche, Bremen (DE)
Jaco Kraan, Rotterdam (NL)
Stefano Papa, Urbino (IT)

Website:
www.cytometry2008.eu

Congress and Exhibition:
MCI – Berlin Office / Congress Partner GmbH
Markgrafenstr. 56
D-10117 Berlin
Tel.: +49 (0)30 20 45 90
Fax: +49 (0)30 20 45 95 0

Abstracts:
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Günther-Wagner-Allee 13
D-30177 Hannover
Fon: +49-511-542 76-0
Fax: +49-511-542 76-30
E-Mail: office@pharmaservice.de

OS-1: Scientific Opening Session: Epidemiological diseases with tremendous impact in Africa

OS-1-1

Adapted technologies and new concepts for diagnosis of three major diseases in Africa: Malaria, tuberculosis and HIV/AIDS

Göhde W.[1]
[1]*University of Münster, Radiobiology and Experimental Cell Research, Münster, Germany*

The management and successful therapy of the three major diseases in developing countries, Malaria, Tuberculosis and HIV/AIDS, depends on easy and reliable diagnosis.
For many years experts in the developed world made people in Africa and other less developed areas believing that those techniques and procedures coming from industrialised countries would be helpful in their areas. The outcome of these mistakes was, and partially still is a tremendous waste of money, human resources and, in addition, this politics damaged the hope of millions of patients. Alternatives are possible.
The 100 year old „gold standard" in malaria diagnostic Giemsa light microscopy is not reachable for the majority of patients in Africa. Giemsa needs laboratories of certain standards, electricity, cool chain for the supply of fresh reagents and trained lab technicians. The existing infrastructure does not support these needs.
The number of tuberculosis cases is increasing in most African countries by 5 - 7% every year besides all efforts and money. Tuberculosis is the disease of the poor. The most often used diagnostic technique, the Ziehl-Neelsen light microscopy is not very sensitive, needs high level of laboratory infrastructure and trained technicians. All this is not available in remote areas.
The solution for a substantial improvement, which we offer, is the use of the highly sensitive auramine technique. We overcame the problems associated with the high price of fluorescent microscopes, need of electricity and trained technicians by introduction of a battery driven fluorescent microscope at total costs of less than 1.000,00 Euro. The same microscope is used for our 30 sec malaria test as well for the auramine Tb-test.
In the case of HIV/AIDS very effective treatment regimen have been developed during the last few years. All of them are based on a life long monitoring of the immunostatus by counting the CD4 positive lymphocytes respectively the determination of the percentage of the CD4 positive lymphocytes from all lymphocytes.
Based on heavy financial and personnel support from my university we could in collaboration with Partec develop and introduce new protocols and robust and affordable technique for CD4 and CD4% monitoring. This development did allow to reduce the test costs from U$ 40,- (according to WHO figures in 2001) to U$ 2,-. This was a breakthrough for many patients and a change of health politics in many countries.
Meanwhile, more than 800 devices could be installed in developing countries assisting sufficient treatment for more than 600.000 patients this year.
All three developments are in use today. For the acceptance of these „adapted technologies for remote areas" it is abolutely indispenseable that the performance and quality of results must be at least as good as for the „high-tech-counterpart".

OS-1-2

HIV/AIDS, Tuberculosis and Malaria: three killer diseases ; is there any hope for African countries ?

Traoré Y.[1]
University of Ouagadougou; Bobo-Dioulasso, Burkina Faso

AIDS, Malaria and Tuberculosis are known to be the most three killer diseases in African countries. All three infections are related to socio economic conditions in which poverty increases the risks and consequences due to these infections at individual and collective level. On the other hand, when people are infected, they

are more likely at risk of becoming poor. Most of the countries implemented National Programs based on early diagnosis, appropriate treatment and prevention.

Diagnostic tools are available for Malaria, Tuberculosis and HIV infection and they can tremendously improve the outcome of these infections. HIV patients monitoring in terms of CD4+ T lymphocytes counting are more and more available and affordable due to Partec Essential Healthcare solutions. As an example, by offering a CD4 count at 2 USD using a CyFlow, thousands of HIV patients in several countries were able to have access to this measurement as quick as it is scheduled instead of waiting up to three months. In the same time the end price to be paid by the patients was divided by five to ten.

Treatment of Malaria and Tuberculosis are easy and effective when diagnosis is early made. This can be reached by education in order to sensitise population to “warning clinical signs” leading to consultation in medical centres. Even for HIV infection the number of patients accessing to ARVs is still limited to less than 30% of the needs, increasing efforts are observed.

Prevention strategies for the three diseases should be combined with the improvement of socio economic conditions. To reach acceptable results, not only individual behaviour should be addressed but also high commitment at political level.

S-1: Cytometry in Microbiology and Biotechnology

S-1-3

Application of flow cytometry to the analysis of the gut microbial ecosystem

Blaut M.[1]
[1]*German Institute of Human Nutrition, Potsdam-Rehbrücke, Nuthetal, Germany*

The microbial community inhabiting the human gastrointestinal tract exerts profound effects on gastrointestinal function and health. For example the formation of short chain fatty acids from non-digestible carbohydrates plays an important role in controlling cell growth and differentiation of colonic epithelial cells. Moreover, the intestinal microbiota has a barrier effect against pathogenic bacteria, but it has also been implicated in the formation of compounds that may be deleterious to the host. There is nevertheless little knowledge on whether and how the composition of the intestinal affects host physiology. This is partly due to the complexity of the interactions between gut microorganisms, host and diet, and partly to the variability between human individuals in respect of the composition of the intestinal microbiota. Considerable progress has been made in improving the knowledge of the microbial diversity of the human intestinal tract. This increase in knowledge is based on the isolation and characterisation of a considerable number of previously un-described bacterial species and on the retrieval of a large number of novel 16S rRNA sequences in a culture independent approach.

S-1-4

A new fluorescent in vivo detection method for polyphosphate accumulating bacteria

Günther S.[1], Röske I.[2], Harms H.[1], Müller S.[1]
[1]*UFZ-Centre for Environmental Research Leipzig-Halle, Department of Environmental Microbiology, Leipzig, Germany,* [2]*Dresden University of Technology, Dresden, Germany*

Although wastewater treatment plants with Enhanced Biological Phosphorus Removal (EBPR) represent a so called state-of-the-art technology for phosphate removal, those processes are known to be prone to regular breakdown. The reason is lack of knowledge about structure and function of the bacterial community involved in the phosphorus removal process. Most of the bacteria are still not cultivable as pure cultures which is the main reason why their function during the waster water treatment process was either not or under-estimated. However, function and phylogeny of the mostly uncultivable phosphorus accumulating organisms (PAOs) need to be clarified to enable for process optimization of wastewater plants. Therefore, flow cytometry is used as fast and convenient approach to grasp uncultivable bacteria capable of polyphosphate accumulation within highly heterogeneous communities. A novel fluorescent staining technique to detect quantitatively polyphosphate granules on the individual level was developed. The technique is based on detection of the bright fluorescence of the cation chelating antibiotic tetracycline, which binds to polyphosphate granules containing divalent cations.

S-1-5

Impact of culture conditions on the surface marker profile and other properties of stem cells from amniotic membrane and adipose tissue

Peterbauer A.[1], Stadler G.[2], Hennerbichler S.[1], Lindenmair A.[2], Aberl J.[1], Leutgöb G.[2], van Griensven M.[2], Gabriel C.[1], Redl H.[2], Wolbank S.[1]
[1]*Red Cross Blood Transfusion Service of Upper Austria, Linz, Austria,* [2]*Ludwig Boltzmann Institute for Experimental and Clinical Traumatology, Vienna, Austria*

Aims: Amniotic membrane and adipose tissue are promising sources for cell-based therapies. Both of them

are abundantly available and raise no ethical concerns. Adipose tissue contains mesenchymal stem cells so-called adipose derived stem cells (ASC), whereas in amniotic membrane two cell types are found: human amniotic mesenchymal stromal cells (hAMSC) and human amniotic epithelial cells (hAEC). We aimed to improve the proliferation capacity and the differentiation potential towards the adipogenic and osteogenic lineage of these cells while maintaining their typical surface marker expression profile and their immunomodulatory properties. With regard to future application of these cells in humans, we emphasized our efforts on using animal-free products for cell expansion including platelet lysate (PL).
Methods: ASC were isolated by collagenase digestion. hAMSC and hAEC isolation was accomplished by separate digestion in collagenase and trypsin, respectively. Several media compositions containing growth factors such as epidermal growth factor, platelet-derived growth factor or vascular endothelial growth factor were applied during expansion. Additionally, PL was used to substitute fetal calf serum (FCS). Cultures were characterized for their surface marker expression profile, proliferation capacity and immunomodulatory properties. Different media for adipogenic and osteogenic induction were applied. Subsequently, adipogenic differentiation was demonstrated by Oilred O staining while osteogenic differentiation was detected by von Kossa or Alizarin Red staining.
Results: ASC, hAMSC and hAEC homogenously expressed typical mesenchymal and embryonic stem cell markers. CD49d was observed only in the two mesenchymal sources but was hardly expressed in hAEC. Furthermore, we demonstrated a dramatic change of surface antigen expression during cultivation of hAEC with an up-regulation of mesenchymal markers and a concomitant decrease of embryonic markers upon in vitro propagation. This phenotypic shift was associated with reduced osteogenic differentiation. All three cell types were able to inhibit the proliferation of activated peripheral blood mononuclear cells in vitro.
Addition of growth factors to the expansion media significantly increased the proliferation potential of the cells. Similar effects were achieved by substituting FCS by PL which in addition preserved the differentiation capacity and did neither alter the surface marker expression profile nor the immunomodulatory properties of the cells investigated.
Conclusions: Platelet concentrate is a precious raw material for producing culture supplements of human origin containing high concentrations of growth factors able to support cells from adipose tissue and amniotic membrane during in vitro propagation while maintaining their phenotypic properties.
Acknowledgments: HIPPOCRATES (NMP3-CT-2003-505758), Lorenz Boehler Fonds, NoE EXPERTISSUES (NMP3-CT-2004-500283).

S-1-6

Characterization of mesenchymal stem cells isolated from human umbilical cord
Moretti P.[1], Hatlapatka T.[1], Majore I.[1], Scheper T.[1]
[1]*Leibniz Universität Hannover, Institut für Technische Chemie, Hannover, Germany*

The increasing demand for human mesenchymal stem cells (hMSCs) for cell-based therapies and tissue engineering leads to tremendous research effort to find novel sources as well as derivation methods for MSCs. Due to its easy availability and, compared with the bone marrow, higher frequency of MSCs, the human umbilical cord presents a promising alternative source for these cells. The aim of our investigation is to develop a biotechnological process for the extensive expansion of hMSCs.
In this work we detail a method for the isolation of hMSCs from human umbilical cord matrix and the in-vitro expansion of the cells under optimized conditions in a-MEM containing 10% human serum. The morphology, the proliferation potential as well as the surface antigen expression was investigated. For the latter the cells were analyzed on the single cell level by flow cytometry. Three-colors protocols were developed to allow the simultaneous analysis of hematopoietic and common leukocyte markers (CD34, CD45), marker related to adhesion (CD44) and mesenchymal stem cell markers (CD90, CD105, CD73). After isolation the phenotype of the cells was monitored over long term cultivations.
The isolated cells are plastic adherent presenting a fibroblastic morphology. Cells express typical mesenchymal stem cell markers as CD73, CD90, CD105 and CD44 and lack expression of CD45 and

CD34. The expression remains unchanged throughout all examined passages (7). Furthermore, the results of our investigation indicate that a fast and efficient expansion of hMSC is carried out best when the culture medium is supplemented with HS rather than FCS. Presently, the differentiation potential of the isolated cells to osteoblasts and adipocytes is investigated.

S-1-7

VioBlue™ A novel violet-excited dye for multicolor flow cytometry

Herber M.[1], Weber-Lohmann S.[1], Büscher M.[1], Krauthäuser S.[1]
[1]*Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany*

Aim: The importance of new fluorescent dyes for flow cytometry has increased since detection of more than 4 colors is feasible on commercial analyzers. Simultaneously, the number of monoclonal antibodies conjugated to fluorochromes for excitation with 488nm or 635nm lasers is expanding. However, for flow cytometers equipped with 405nm laser the choice of antibody-fluorochrome conjugates is limited since there are only a few suitable dyes.
Here we present a novel violet-excited fluorochrome, VioBlue, to increase the availability of reliable dyes for multicolor analysis. This new dye provides an additional option to transfer staining of abundant antigens from the 488nm and 635nm excitation lines to the violet laser. Furthermore, we demonstrate the applicability of CD45-VioBlue conjugates to be used as a trigger in automated acquisition and analysis of leukocytes.
Methods: Several monoclonal antibodies specific for human or mouse leukocyte antigens were coupled to the new dye and compared to corresponding commercially available Pacific Blue antibody conjugates. Human peripheral blood mononuclear cells or mouse splenocytes were stained with the VioBlue and Pacific Blue antibody conjugates and analyzed on a MACSQuant flow cytometer. The MACSQuant Analyzer is equipped with 488nm, 635nm laser and a 405nm violet laser diode in combination with a 450/50 bandpass filter for detection of violet dyes. Stain Indices were used as a measure to determine the quality of staining for each antibody conjugate. For automated acquisition and analysis the MACSQuant application “Express mode” was utilized based on CD45-VioBlue staining.
Results: The VioBlue conjugated monoclonal antibodies showed similar or even better staining intensities compared with the Pacific Blue conjugates using violet diode excitation on MACSQuant. Comparison of the relative brightness of VioBlue to FITC revealed equal or only slightly lower staining of the respective antibody conjugates. Furthermore, CD45-Vioblue can be used as a trigger in the violet channel for all applications with CD45 positive target cells. This gating strategy is realized in the automated acquisition and analysis process “Express mode” on MACSQuant. The “Express mode” uses an automated CD45-Trigger in the violet channel in order to define the leukocyte population and to eliminate debris.
Conclusion: VioBlue is a novel fluorochrome, which expands the repertoire of violet-excited dyes. It provides equally bright staining results compared to Pacific Blue and similar or slightly lower Stain Indices compared to FITC. VioBlue antibody conjugates represent an attractive alternative for densely expressed antigens. Furthermore, VioBlue conjugated CD45 antibody allows reliable leukocyte labeling, which serves as basis for automated acquisition and analysis on MACSQuant.

S-1-8

Label-free flow cytometry on a microfluidic chip based on native fluorescence

Beck M.[1], Kiesel P.[1], Bassler M.[1], Johnson N.M.[1], Schmidt O.[1]
[1]*Palo Alto Research Center, Palo Alto, United States*

The detection and characterization of single cells without the need for sample preparation is highly desirable. Flow cytometry based on native fluorescence spectroscopy is a promising approach that does not require specific binding or tagging of the analyte. However, the variety of cells is large compared to the number of basic molecular building blocks. Therefore, the fluorescence spectra of different species are often

very similar, and sophisticated detection methods are required to reveal differences. The specificity of this approach can be improved by implementing high spectral resolution and using multiple excitation wavelengths.
We have developed a compact platform that combines a microfluidic quartz channel with chip-size wavelength-selective detection which records the fluorescence of particles as they traverse the channel. The interaction between the excitation light and the analyte is enhanced by anti-resonantly guiding the excitation light within the analyte-containing fluid. We have recorded the intrinsic fluorescence of single cells (e.g., yeast, e-coli and BT) as they transit the detection area even at high speed. Simultaneously monitoring total intensity and spectrally-resolved emission yields accurate spectra for particle discrimination. Knowing the particle speed and the physical dimensions of the observation window, we are able to determine particle positions with microscopic (<10 microns) resolution. A novel modulation technique allows us to achieve a high signal-to-noise ratio even at high particle speeds. Our platform can readily incorporate both the detection of scatter signals as well as cell sorting techniques to enable on-the-chip characterization and sorting of untagged cells.

This work is partially funded under ONR contract N00014-05-C-0430 and by the Alexander-von-Humboldt-Foundation.

S-2: Tissue Analysis and Imaging

S-2-1

Studying Intracellular transport using Correlative Light Electron Microscopy

Verkade P[1]
[1]*School of Medical Sciences, Wolfson Bioimaging Facility, University of Bristol, University Walk, BS8 1TD Bristol, United Kingdom*

Since the availability of Green Fluorescent Protein for life science research there have been an overwhelming number of studies using live cell imaging. However in some cases the resolution of the light microscope is limiting in answering the scientific question. For those instances, electron microscopy has always been the method of choice. As a consequence, in recent years there has been an increasing interest in Correlative Light Electron Microscopy (CLEM) techniques. This technique combines the dynamic images of the light microscope with the high resolution of the electron microscope. However, all existing CLEM techniques lack a good time resolution (>30 sec.), a good morphological preservation, or both. Clearly, these drawbacks make it very difficult to properly capture most processes taking place inside the cell. With the internalization of EGF coupled to Alexa488 and 10nm gold as markers, a technique / machine is described where the time between imaging and fixation is reduced to maximum 5 seconds with optimal ultrastructural preservation. This method is based on high pressure freezing and should give us the tools to unravel intracellular processes that can be observed by life cell imaging but are too rare and fast to be picked up by routine EM methods for study at high resolution. The detection of the markers used for CLEM, which should be both fluorescent and electron dense, in the electron microscope is another place for possible improvement. The use of High Angle Annular Dark Field HAADF STEM for this is one of our research interests.

S-2-10

Endothelial progenitor derived cells express the SR-B1 scavenger receptor and take up high density lipoprotein as ligand (A study using correlative microscopy, DAB photoconversion and electron tomography)

Neumüller J.[1], Meißlitzer-Ruppitsch C.[1], Röhrl C.[2], Stangl H.[2], Pavelka M.[1]
[1]*Medical University of Vienna, Center for Anatomy and Cell Biology, Dept. of Cell Biology and Ultrastructure Research, Vienna, Austria,* [2]*Medical University of Vienna, Center for Physiology and Pathophysiology, Institute of Medical Chemistry, Vienna, Austria*

Background: Many working groups could show the protective role of high density lipoprotein (HDL) against the development of arthrosclerosis and coronary heart diseases. This beneficial effect is due to reverse cholesterol transport which removes excess of cholesterol from peripheral tissues and delivers it back to the liver. In many cell types, HDL is taken up by the scavenger receptor SR-B1. In this study we demonstrate the presence of SR-B1 in endothelial progenitor derived cells (EPDCs) and the internalization pathway using light and electron microscopy including electron tomography (ET).
Material and methods: EPDCs were generated from human cord blood CD133+ cells using fibronectin-coated culture flasks and conditioned medium containing VEGF driving cell differentiation toward endothelial cells (1). The expression of FITC-labeled SR-B1 and the uptake of Cy3-labeled HDL via this receptor were investigated in EPDCs using confocal laser scanning microscopy (LSM) and subsequent diaminobenzidine (DAB) photoconversion. The principle of this technique, originally described by Maranto in 1982 (2) has been evaluated for a variety of cell biological applications (3). It involves the irradiation of fluorolabeled cellular structures with the excitation wavelength of the respective fluorophor resulting in oxygen radical generation and conversion into an insoluble electron-opaque osmophilic DAB polymer that allows localizing the fluorolabeled structures by transmission electron microscopy (TEM). Labeled organelles were investigated in 200-300 nm semithin sections and visualized in 3D using ET. This technique include

acquisition of tilt series, alignment procedures and reconstruction of the sectioned volume by weighted back projection resulting in virtual slices from which 3D models could be established.
Results and conclusion: Immunostaining of EPDCs with a polyclonal antibody against SR-B1 receptor and a FITC-conjugated 2nd step antibody could clearly demonstrate its presence at the cell membrane and in caveolae using LSM and TEM. The internalization of Cy3 labeled HDL was also shown by LSM. In TEM, HDL uptake could be impressively demonstrated in endosomal compartments and in 1-2 cisterns of the Golgi apparatus as well as in the trans-Golgi-network where it exhibits a nodular appearance which could be visualized in 3D by ET. This study underlines that the combination of LSM, DAB-photoconversion and ET is an appropriate tool for correlative microscopy.
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S-2-11

Concentration measurement of fluorescent solutions by slide based cytometry
Pierzchalski A.[1], Marecka M.[1], Müller H.-W.[2], Bocsi J.[1], Tárnok A.[1]
[1]University of Leipzig, Pediatric Cardiology, Leipzig, Germany, [2]Sensovation AG, Stockach, Germany

Background: Flow cytometers are built for particle measurements. In principle, concentration measurement of a homogeneous solution is not possible due to the lack of a trigger signal. By contrast to FCM slide based cytometry systems could act as tools for the measurement of concentrations using volume defined cell counting chambers [1]. These chambers enable to analyze a well defined volume. Sensovation AG (Stockach, Germany) introduced a new automated imaging system that combines imaging with cytometric features analysis. Aim of the study was to apply this imaging system to quantify the fluorescent molecule concentrations.
Methods: The innovative Lumisens (Sensovation AG) slide-based technology based on fluorescence digital imaging microscopy was used. The instrument is equipped with an inverted microscope, blue and red LEDs, double band pass filters and high-resolution cooled 16-bit digital camera. The instrument was focussed on the bottom of 400µm deep 6 chamber slides (IBIDI GmbH, Martinsried, Germany) or flat bottom 96 well plates (Greiner Bio One GmbH, Frickenhausen, Germany). Fluorescent solutions were imaged under 95-99% pixel saturation in a broad concentration range (FITC: 0.0002-250 µg/ml, methylen-blue (MethB): 0.0002-250 µg/ml). Exposition times were recorded. Images were analysed by the iCys (CompuCyte Corp., Cambridge, MA, USA) image analysis software with the phantom contour function. Relative fluorescence intensities were calculated from mean fluorescence intensities per phantom contours divided by the exposition time.
Results: Solution concentrations could be distinguished over a broad dynamic range of 3.5 to 5.5 decades log (range FITC: 0.0002-31.25µg/ml, MethB: 0.0076-31.25µg/ml)) with a good linear relationship between dye concentration and relative fluorescence intensity. The minimal number of fluorescent molecules pro pixel as determined by the mean fluorescence intensity and the molecular weight of the fluorochrome were about 800 molecules FITC and ~2.000 MethB. T
Conclusion: The novel slide-based imaging system is suitable for detection of fluorescence differences over a broad range of concentrations. This approach may lead to novel assays for measuring concentration differences in cell free solutions and cell cultures e.g. in secretion assays.
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The authors thank the AiF, Berlin, Germany for financial support.

S-2-12

Signal protein translocation in differentiating human neural progenitor cells: Generation of quantitative data for a systems biology approach from confocal 3D image stacks

Bader B.M.[1], Weiss D.G.[1]
[1]*University of Rostock, Institute of Biological Sciences, Cell Biology and Biosystems Technology, Rostock, Germany*

Quantitative image analysis is an advancing field in systems biology since it provides the data needed for modeling spatio-temporal changes caused by signalling cascades which include protein movements and transformations of the cellular 3D architecture. Signalling networks such as the canonical Wnt-pathway regulate cellular differentiation steps controlled by TCF-dependent transcription. The activation of transcription depends on β-catenin translocation into the nucleus. We quantified the translocation of proteins of the Wnt cascade between different cellular compartments during the early differentiation phase of human neural progenitor cells VM 197 by semi-automatic quantitative analysis of confocal image stacks using Imaris™ software (Bitplane). To investigate the influence of Wnt-signaling on both, the cellular fate and β-catenin localization, the cells were treated with either activators or inhibitors of the Wnt signalling cascade: SB216763 an inhibitor of glycogen synthase kinase-(GSK)-3β, WIF-I (Wnt inhibitory factor-I) and the canonical Wnt-antagonist Dickkopf-1 (Dkk1). Wnt3a, a stimulator of the canonical Wnt-pathway, was used to investigate the upstream effects of this pathway. The results show that the nuclear β-catenin concentration increases pari passu with differentiation into neuronal and glial phenotypes. Treatment with SB216763 and Wnt3a enhanced this effect, whereas Dkk1 and WIF-I treatment delayed it. One major aspect of this work was the comparison of Wnt pathway effects between neurons and glia cells. The continuous quantification of the neuronal phenotype during the first days supported the evidence that the differentiation in these cells is indeed controlled by the Wnt-pathway since it is accelerated or delayed by Wnt-agonists and antagonists, respectively.
This precise quantification of fluorescence signals in 3D volumes correlates with neuronal phenotype development and, therefore, provides a means to investigate also other protein translocation processes between cellular compartments. Since the method is applicable to quantitatively describe different phenotypes the obtained data are well-suited for modeling cellular differentiation mechanisms which are correlated with and possibly caused by spatio-temporal changes in signal protein distribution.

Supported by DFG GRK 1387 dIEM oSiRiS

S-2-13

Cell based cytometry using wide field microscopy

Malkusch W.[1]
[1]*Carl Zeiss ISG, PM, Hallbergmoos, Germany*

Introduction: A huge amount of cells is measured very fast with flow cytometry (FCM). But usually the direct link of parameter data to individual cells is missing. The AxioVision Scanning Fluorescence Microscope (AV SFM) will fill this gap using cytometry-like results to provide the missing direct reference of the data to the originating cells.
Methods: Statistically sufficient cells from cytospins will be acquired in multiple fluorescence channels (AV MosaiX) with a digital camera (AxioCam MRm) on a fluorescence microscope (AxioImager). The images will be measured with AV software. All cell results are part of a single data table. The AV SFM module is linking the data with the original image. Various distribution types may be created (histogram, scatter plot, gallery, and gated data table) to isolate cells as an image together with the connected data.
Results: The results table of the AV SFM also holds the cell coordinates for the various channels and the window coordinates for each single cell in addition to the raw data and the derived parameters. In experiments on beads preparations with 8 different populations all 8 classes could be isolated distinctly

using a 3 channel setup in image acquisition and cell separation. Implementation of shading correction patterns and wave-length dependent Z- corrections for the filter sets are a pre-requisite for this separation. In experiments with CD45-CK and CD3-CD8 cells 2 parameters were distributed in scatter plots. The rare events selected via gatings were visualized in a cell gallery. There is also a feedback of each cell in the gallery to its position in the digital slide.
Conclusions: AV SFM seems to be a reliable alternative method to LSC and FCM, as shown in previous papers. In order to find and measure rare cells AV SFM with digital slides is a beneficial extension of conventional fluorescence microscopes. In order to consolidate the consistency of these results further investigations will be performed.

S-2-14

Cellular and humoral immune response to open heart surgery in children is modulated by methylprednisolone

Bocsi J.[1], Hänzka M.-.C.[1], Hambsch J.[1], Schneider P.[1], Tárnok A.[1]
[1]*Heart Center, University of Leipzig, Pediatric Cardiology, Leipzig, Germany*

Introduction: Postoperative effusions, oedema and capillary leak syndrome in children after cardiac surgery with cardiopulmonary bypass (CPB) constitute considerable clinical problems. Acute immune reactions as a response to cardiovascular surgery with CPB are held responsible for these adverse effects. In order to reduce overshooting immune response glucocorticoids are administered pre- and perioperative. In a retrospective study we investigated the modulation of the humoral and cellular immune response by methylprednisolone (MP).
Methods: This study was carried out with children undergoing cardiac surgery without (MP-, n = 10) and with MP administration before CPB (MP+, n = 23, dose: 5-20 mg/kg body weight). EDTA anticoagulated blood was obtained 24 h preoperatively, after anaesthesia, CPB begin, CPB end, 4h, 24h, 48h after surgery, at discharge and at out-patient follow-up (9.1+7.0 month after surgery). The humoral immune response was monitored by serum levels of immunoglobulins, complement, cytokines and chemokines, soluble adhesion molecules and receptors as well as clinical chemistry parameters such as differential counts. The cellular immune reaction was monitored by flow cytometry. Different 3- and 4-color antibody panels were used to determine differential blood count, lymphocyte subsets and the degree of activation of various leukocyte subpopulations. [1]
Results: The patients follow up was uneventful and effusion volume, intensive care unit and hospital days were in the same range with and without MP administration. Release of the proinflammatory cytokines IL-6 and IL8 was reduced and that of the anti-inflammatory cytokine IL-10 upregulated by MP. Significant peri- und postoperative increase of circulating neutrophils and monocytes as inflammatory reaction to surgery and CPB contact was detected in both groups. However, infiltration of these cells was delayed with MP. Under both treatment modalities the levels of activation (LFA-1 and Mac-1 expression) were similar for MP+ and MP- and lower than before surgery as found earlier [2].
The CD4+ and CD8+ T-lymphocyte cell count decreased and the T4/T8 ratio was lower with MP treatment. The major difference was found for B-lymphocytes: With MP their cell count increased significantly after surgery but remained constant in the MP- group.
Conclusions: MP treatment decreased the proinflammatory effect of the CPB surgery without clinical detectable differences during surgery and at outcome. MP shows antiinflammatory effect on cellular and humoral level, but without easily detectable clinical advantages.
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The authors thank the Deutsche Stiftung für Herzforschung, Frankfurt, Germany for financial support.

S-3: Cancer Biology of Solid Tumors

S-3-15

Role of EGF receptor tyrosine kinases in breast cancer. A biophysical approach
Szollosi J.[1], Vereb G.[1], Barok M.[1], Palyi-Krekk Z.[1], Nagy P.[1]
[1]University of Debrecen, Department of Biophysics and Cell Biology, Debrecen, Hungary

The ErbB2 (HER2) protein is a crucial member of the EGF receptor (ErbB) family of transmembrane receptor tyrosine kinases. Although no direct ligand has yet been assigned to ErbB2, recent biochemical and biophysical evidence suggests that this protein operates as a shared receptor subunit with other ErbB proteins. Its medical importance is supported by the facts that it is frequently overexpressed in breast and other cancers. Humanized antibodies against ErbB2 (i.e. Herceptin) have been introduced into clinical practice and were found to have cytostatic effect in ~40% of ErbB2 positive breast tumors. Our working hypothesis is that the expression levels of ErbB kinases, their interactions and activity within multimolecular complexes will determine the outcome of ErbB2 directed therapy. We used Herceptin resistant (JIMT-1) and sensitive (SKBR-3) cell lines in order to demonstrate the importance of association pattern ErbB molecules with each other and with integrins, CD44 and lipid rafts. Combination of various forms of flow and image cytometric FRET methods revealed distinctive expression and association pattern of ErbB receptor tyrosine kinases on the surface of various cancer cell lines sensitive or resistant to trastuzumab. Simultaneous application of image cytometric FRET methods based on donor and acceptor photobleaching provided a useful two sided FRET approach revealing a unique co-association pattern of integrins, CD44 and ErbB2 on the surface of tumor cells. Fluorescence correlation spectroscopy demonstrated stable co-association of ErbB2 and ErbB3 on both trastuzumab sensitive and resistant cell line. We also studied the role of antibody mediated cellular cytotoxicity (ADCC) using JIMT-1 cell xenografts in SCID mice. Unexpectedly, trastuzumab was able to inhibit the outgrowth of macroscopically detectable xenograft tumors for up to 5-7 weeks. This effect was further enhanced when 4-methyl-umbelliferrone (4-MU) was added along with trastuzumab treatment. 4-MU inhibited the synthesis of hyaluronan, which binds to CD44, thereby hindered the development of the glyco-calix surrounding the tumor cells. Interestingly trastuzumab treatment highly decreased the number of circulating tumor cells even when the tumor became completely resistant to trastuzumab treatment.

S-3-16

Initial mechanisms triggering ErbB mediated signaling - implications for efficient targeted therapy
Diermeier-Daucher S.[1], Friedländer E.[1], Vereb G.[2], Brockhoff G.[1]
[1]Institute of Pathology, University of Regensburg, Regensburg, Germany, [2]Department of Biophysics and Cell Biology, University of Debrecen, Debrecen, Hungary

Shaping well defined three-dimensional structures upon ligand binding by specific lateral ErbB-receptor interaction is a crucial event that defines specific intracellular signaling and in turn causes specific cellular response. PTB and SH2 docking sites selectively recruit downstream enzymes and adaptor proteins. However their formation largely depends on the explicit coexpression pattern of ErbBs and the specificity / generality of ligand-receptor binding. These two parameters predominantly determine both the amplitude and the quality of intracellular signals. Hence, disregulated signal transmission across the cell membrane contributes or even causes cellular malignancy represented by e. g. uncontrolled cell proliferation.
Here we show ligand (growth factor, antibody) specific induction and disruption of receptor interaction and cross-activation quantitatively analyzed by FRET and multicolor flow cytometry. ErbB receptor coexpression patterns in breast cancer cell lines were taken into account when activation of intracellular signaling of key molecules was analyzed. Cell cycle kinetics were dynamically assessed via flow cytometry. Cell barcoding based multicolor flow cytometry was applied for the analysis of intracellular signaling.
We demonstrate that targeting the same receptor using reagents (ErbB specific antibodies) with different

binding specificity induces ligand specific assembly and disassembly of ErbB receptors. Reagents eliciting opposed molecular mechanisms however can mutually enhance each others antiproliferative effect. Treatment efficiency can be enhanced by modulating the ErbB receptor coexpression/interaction pattern. The understanding of coexpression and ligand dependent initial lateral receptor signaling provoking specific patterns of intracellular signaling will facilitate highly specific treatments to curb uncontrolled cell proliferation of tumor cells.

S-3-17

Three-level based analysis of c-erbB receptor tyrosine kinases in breast cancer

Sassen A.[1], Rochon J.[2], Hofstaedter F.[1], Schwarz S.[3], Brockhoff G.[1]
[1]University of Regensburg, Department of Pathology, Regensburg, Germany, [2]University of Regensburg, Center for Clinical Studies, Regensburg, Germany, [3]University of Erlangen, Department of Pathology, Erlangen, Germany

The HER (human EGFR related) family of receptor tyrosine kinases (HER1/EGFR (epidermal growth factor receptor)/c-erbB1, HER2/c-erbB2, HER3/c-erbB3 and HER4/c-erbB4) shares a high degree of structural and functional homology. It constitutes a complex network, coupling various extracellular ligands to intracellular signal transduction pathways resulting in receptor interaction and cross-activation (Slamon et al., 1989). One of the most famous family members is c-erbB2, which is an important prognostic marker in breast cancer in case of dysregulation as a result of gene amplification and protein overexpression. Since the clinical outcome with regard to total c-erbB receptor state remained largely unknown, we investigated c-erbB1-c-erbB4, at both the DNA and the protein level, using fluorescence-in-situ-hybridisation (FISH) probes targeted to all four receptor loci and also immunohistochemistry (IHC) in tissue microarrays derived from 278 breast cancer patients. We retrospectively found c-erbB3 gene amplification with a univariate negative impact on disease-free survival, whereas c-erbB4 amplification showed a positive trend in overall and disease-free survival (Sassen et al., 2008).
IHC revealed conflictive information concerning the impact of c-erbB3 and c-erbB4 on patient survival. Therefore, further information with regard to regulation of transcription as a critical step between gene status and receptor expression is needed. We designed DIG-labeled DNA probes and investigated RTK mRNA expression in the same patient cohort.
At the current state, in-situ-hybridization is expected to be a powerful and specific tool for c-erbB mRNA detection in breast cancer and to illuminate oppositional and not comprehensible experimental results. Overall, the simultaneous quantification of c-erbB3 and c-erbB4 receptor mRNA by ISH might enable the rendering of a more precise stratification of breast cancer patients by providing additional prognostic information. Explorative and prospective studies on all c-erbB receptors are required for a precise evaluation of their potential use for specific therapeutic targeting with respect to individualized therapy.

S-3-18

Influence of anti-her2/neu therapy on Gene analysis of circulating epithelial tumor cells (CETC) under the influence of anti-her2/neu therapy

Pachmann K.[1]
[1]Friedrich Schiller University Jena, Clinic for Internal Medicine II, Department of Experimental Hematology and Oncology, Jena, Germany

The amplification of the her2/neu gene coding for the epithelial growth factor receptor ErbB2 implies a poor prognosis in breast cancer. However, one of the antibodies directed against an extra cellular domain, trastuzumab, has shown to improve the prognosis of these patients in the metastatic as well as the adjuvant situation. In the adjuvant setting the antibody is supposed to act on the remnant circulating tumor cells. Therefore we have investigated the influence of trastuzumab on the CETCs detected by the MAINTRAC®

approach.
CETCs were monitored during trastuzumab treatment after adjuvant chemotherapy and the composition of subpopulations carrying different amounts of her2/neu gene copies analyzed by FISH.
CETCs decreased continuously in part of the patients during 1 year of trastuzumab treatment and this has been shown to implicate a relapse free survival. However, in some patients CETCs started increasing in spite of trastuzumab treatment, and this was due to an increase in the population of cells, carrying more than 10 gene copies of her2/neu per cell.
Thus the resistance of her2/neu positive tumors to the treatment with trastuzumab seems in part to be due to the evolution of a population of cells with very high her2/neu expression.
This raises the question, whether in patients with increasing CETCs in spite of conventional trastuzumab therapy higher doses of this antibody might be able to prevent resistance and thus relapse.

S-3-19

Inhibition of insulin-like growth factor type I receptor 1 (IGFR1) gene, but not epidermal growth factor receptor (EGFR) neither Her-2/neu orphan receptor genes, induced phenotype changes relevant to increased tumor susceptibility to immune system in A549 human lung cancer cells

Kopinski P.[1], Pólgesek E.[1], Jaworska A.[1], Dancewicz M.[2], Szablowska K.[1], Kowalewski J.[2]
[1]Collegium Medicum, Nicolaus Copernicus University, Chair of Gene Therapy, Bydgoszcz, Poland,
[2]Collegium Medicum, Nicolaus Copernicus University, Chair of Thorax and Tumor Surgery, Bydgoszcz, Poland

Background: Growth factors as IGF-I and EGF, as well as their receptors seem to play an important role in lung carcinogenesis. IGFR1 and EGFR are expressed at remarkable levels on lung cancer cells, participating in tumor cell proliferation and in apoptosis inhibition.
Methods: A549 cell line, model of non-small cell lung cancer, were transfected with small interfering RNA (siRNA) sequences directed against IGFR1, EGFR and Her2/neu gene transcripts. Transfection efficiency was estimated by RT-PCR and flow cytometry (FCM). Additionally FCM was employed to test MHC I, MHC II, B7 co-stimulatory (CD80, 86) molecules, Fas, Fas-ligand, TNFR1, TNFR2 and Bcl-2 family expression, analyzed before and after transfection. Apoptosis was detected by Annexin V staining, TUNEL assay and cell cycle analyses (with propidium iodide, PI, in permeabilized cells).
Results: (median of 7 experiments) A549 transfected with anti-IGFR1 siRNA showed significant ($p<0.05$) increase in CD86 (19 vs 9% cells), MHC I (95 vs 68%), Fas-ligand (57 vs 23%) and Bax (6 vs 4%) expression. Increse in CD80 expression was insignificant. In other experiments, only Fas and Fas Ligand relevant changes were found after si-RNA anti-EGFR transfection. What´s more, increase of expression of other growth factor receptors, as IGFR1 and TNFR2 was found after siRNA dependent EGFR and Her2/neu inhibiotion. Bcl-2 expression (73 vs 26% cells) decline was observed after siRNA anti-IGFR1 transcfection. All receptor gene si-RNA blocking procedures caused significant inrease in cell apoptosis rate and inhibited proliferation.
Conclusions: IGFR1 inhibition in A549 cells caused the range of phenotypic changes relevant to potential stimulation of anti-tumor adopt immunity, including over-expression of MHC I and B7 co-stimulatory molecules. Decline in Bcl-2 intracellular expression could be responsible in part for reported changes. IGFR seems to be a promising target of immune gene therapy in non-small cell lung cancer.

S-3-20

Automated analysis of subcellular patterns in human protein atlas images: Application to identification of potential biomarkers for prostate cancer

Murphy R.F.[1], Glory E.[1], Newberg J.[1]
[1]*Lane Center for Computational Biology, Center for Bioimage Informatics, and Departments of Biomedical Engineering, Biological Sciences and Machine Learning, Carnegie Mellon University, Pittsburgh, United States*

The systematic study of subcellular location patterns is required to fully characterize the human proteome, as well as for identifying potential biomarkers and therapeutic targets associated with disease. The analysis of tens of thousands of expressed proteins for the many cell types and cellular conditions under which they may be found creates a need for automated subcellular pattern analysis. We therefore describe the application of automated methods, previously developed and validated by our laboratory on fluorescence micrographs of cultured cell lines, to analyze subcellular patterns in tissue images from the Human Protein Atlas. The Atlas currently contains images of over 3000 protein patterns in various human tissues obtained using immunohistochemistry. We chose a 16 protein subset from the Atlas that reflects the major classes of subcellular location. We then separated DNA and protein staining in the images, extracted various features from each image, and trained a support vector machine classifier to recognize the protein patterns. Our results show that our system can distinguish the patterns with 83% accuracy in 45 different tissues, and when only the most confident classifications are considered, this rises to 97%. These results are encouraging given that the tissues contain many different cell types organized in different manners. We next applied these subcellular pattern classifiers to images of many different proteins in both normal and cancerous prostate tissue. Comparison of the patterns revealed a set of proteins whose subcellular location appears to change with disease, suggesting that there location (but not necessarily their expression) was a disease marker. Encouragingly, some of these correspond to molecules previously identified as being associated with cancer.

JS-1: Cell Signalling

JS-1-21

The EGF receptor family: Spearheading a merger of signaling and therapeutics

Yarden Y.[1]
[1]*The Weizmann Institute of Science, Department of Biological Regulation, Rehovot, Israel*

Growth factors and their transmembrane receptors contribute to all steps of tumor progression, from the initial phase of clonal expansion, through angiogenesis to metastasis. Hence, the information relay system involved in growth factor signaling provides potential sites for signal interception and tumor inhibition. A relevant example comprises the epidermal growth factor (EGF) and the respective receptor tyrosine kinase, namely ErbB-1/EGFR, which belongs to a prototype signaling module that drives carcinoma development. The extended module includes two autonomous receptor, EGFR and ErbB-4, and two non-autonomous receptors, namely: a ligand-less oncogenic receptor, HER2/ErbB-2, and a kinase-dead receptor (ErbB-3). This signaling module is richly involved in human cancer and already serves as a target for several cancer drugs.
Due to inherent complexity and a large amount of experimental data, we propose a systems approach to understanding ErbB signaling. EGF - to - ErbB signaling is envisioned as a bow-tie configured, evolvable network, sharing modularity, redundancy and control circuits with robust biological and engineered systems. Our work concentrates on system controls, a plethora of negative feedback loops, which include E3 ubiquitin ligases, receptor endocytosis and newly transcribed genes. Because network fragility is an inevitable tradeoff of robustness, systems level understanding is expected to identify therapeutic opportunities for targeting aberrant activation of the network in human pathologies. Specific examples include anti-receptor antibodies, such as Trastuzumab, as well as kinase inhibitors, such as Lapatinib. Mechanisms underlying response to drugs and evolvement of secondary resistance will be discussed.

JS-1-22

Identification and characterisation of drug insensitive stem cells in chronic myeloid leukaemia

Mountford J.C.[1]
[1]*University of Glasgow, Experimental Haematology, Faculty of Medicine, Glasgow, United Kingdom*

The existence of cancer stem cells that sustain disease has been recognised in leukaemia for over a decade. More recently, advances in methods for stem cell recognition have allowed this concept to be investigated in solid tumours and it is now accepted that tumours of many organs, including the brain, breast and prostate, also contain a cancer stem cell population. That this population may sustain and re-populate the disease is of particular relevance in this age of targeted molecular therapy.
The paradigm for targeted therapy and cancer stem cells is Imatinib Mesylate (IM) in Chronic Myeloid Leukaemia (CML). The earliest suggestion that CML arises in a pleuripotent stem cell came in 1951, this was confirmed in the 1990s by gene marking of transplantable leukaemia initiating cells. We and other groups have shown these CML stem cells to be very similar to normal stem cells in their properties and importantly, to be relatively insensitive to the apoptotic effects of IM. This insensitivity raises many questions about the long-term efficacy we can expect from this drug, as IM will destroy the majority of the leukaemic cell load but spare the stem cells which can later re-capitulate the disease. I will discuss the work we are now undertaking that aims to dissect the characteristics and behaviour of these CML stem cells, to discover how to distinguish them from normal haemopoietic stem cells and how to better target this clinically important population of cells.
Work on leukaemia stem cells has resulted in the development of a number of methods for identification and tracking stem cells that can be directly translated into other cancer models. Also, many of the findings may also be of relevance to solid tumours and critically this previous experience may inform the rational design of stem cell directed therapies for many different cancers.

JS-2: Rare Event Analysis and Sorting

JS-2-23

Analysis of rare circulating cells in non-invasive diagnosis

Paterlini Brechot P.[1]
[1]*INSERM, U. 807, Paris, France*

The study of Rare Circulating Cells targets a new biomedical domain susceptible to bring relevant advances in medical care and quality of life. In one ml of blood we count an average number of 10 millions leukocytes, 5 billions erythrocytes but only few “rare cells”. The field of circulating rare cells includes at present: tumor cells, fetal cells, epithelial cells, endothelial cells and stem cells.
Recent molecular and clinical studies have shown that tumor cell invasion may occur very early in tumor development, thus emphasizing the potential importance of specific and sensitive detection of circulating tumor cells (CTC) and circulating tumor microemboli (CTM). The technical challenge in this field consists of finding “rare” tumor cells and being able to distinguish them from epithelial non-tumor cells and from leukocytes.
In the light of new studies, showing that the most invasive tumor cells tend to loose their epithelial antigens (by the Epithelial to Mesenchymal Transition (EMT) process) and that non-tumor epithelial cells can also be present in blood, it appears that a reliable diagnostic identification of CTC and CTM cannot be based on the expression of epithelial-specific transcripts or antigens.
The cytopathological examination of CTC/CTM, sensitively enriched from blood by using ISET (Isolation by Size of Tumor/Trophoblastic cells) will be discussed.
At present, the strategy adopted to detect genetic handicap in unborn children involves the study of foetal cells obtained by amniocentesis or choriocentesis, two methods which are associated with a not inconsiderable risk of miscarriage (2%). An alternative would consist in identifying and isolating the rare foetal cells which circulate in maternal blood. Indeed, these cells provide a source of fœtal DNA which could undergo genetic analysis. However, the number of circulating fœtal cells (CFC) is very small, approximately one or two cells per ml of blood. Among the foetal cells which cross the placental barrier, four types have been identified and studied: myeloid and lymphoid progenitors, erythroblasts and trophoblastic cells (cytotrophoblasts and syncytiotrophoblasts). With respect to myeloid and lymphoid progenitors (CD34 and CD38 positive cells), it has been shown that they may persist in maternal blood for up to 27 years after a pregnancy or miscarriage. For this reason, their isolation is of no value for the prenatal diagnosis of a current pregnancy. However, this problem does not affect erythroblasts and trophoblastic cells.
We have shown the possibility to isolate circulating foetal epithelial cells on the basis of their size, using ISET (Isolation by Size of Epithelial Tumor/Trophoblastic cells). This approach allowed us to develop a non invasive method for prenatal diagnosis of Spinal Muscular Atrophy (SMA) and Cystic Fibrosis. Furthermore, we successfully validated in 2007 the ISET method for non invasive prenatal diagnosis of Spinal Muscular Atrophy.

JS-2-24

Tumor cells and endothelial cells in blood of patients with metastatic carcinomas

Terstappen L.[1]
[1]*Immunicon/ University of Twente, Enschede, Netherlands*

Tumor and endothelial cells dislodged into the blood may represent a biomarker that can be applied across the various carcinomas to assess prognosis and predict or assess response to therapy. To overcome the difficulties associated with the detection of the extremely rare Circulating Tumor Cells (CTC) and Circulating Endothelial Cells (CEC) the CellTracks® technology was developed. This automated sample preparation and analysis system is based on immuno-magnetic enrichment from whole blood followed by immuno-fluorescent labeling and microscopic detection of cells exhibiting a defined phenotype. CTC are defined as

EpCAM+, Cytokeratin 8,18,19+, CD45-, nucleated (DAPI+) intact cells in 7.5 mL of blood and CEC as CD146+, CD105+, CD45-, nucleated (DAPI+) intact cells in 4 mL of blood. Prospective multi-center studies in metastatic breast, colorectal and prostate cancer were conducted and showed that CTC are an independent predictor of Progression Free Survival and Overall Survival. Repeated assessment of CTC in patients undergoing therapy showed to be an effective means to predict outcome and monitor treatment. CEC in these studies were significantly elevated as compared to levels in healthy individuals. Whether this elevation is tumor related or due to vascular damage caused by the treatment is yet unclear.
The number of therapies available to treat patients with recurrent cancer is increasing. As a consequence the oncologist is faced with the challenge which of them will be effective in the individual patient. Determination of the absence or presence of treatment targets on the primary tumor may however be a misrepresentation of the present situation as the original tumor clone is genetically unstable and continues to mutate at a rapid rate constantly giving rise to variants resistant to the particular therapeutic regimen. Presence of therapeutic targets can be assessed on CTC by addition of fluorescently labeled antibodies identifying the targets and or reanalysis of the same cells for amplification of specific genes by FISH. Knowledge of the presence of specific drug related molecular targets on CTC could provide a means to administer a therapy that has a larger chance of success and opens the road toward personalized medicine.

JS-2-25

Single-cell based genomic characterization of tumour cells

Klein C.[1]
[1]*University of Regensburg, Division of Oncogenomics, Regensburg, Germany*

Single disseminated tumour cells (DTC) in bone marrow or tumour cells circulating in the blood (CTC) are increasingly studied because great hope rests in their potential use as markers for disseminated or progressive disease, as tool for therapy monitoring and source for the identification of novel therapy targets. Moreover, analyzing the so far dark period of cancer progression between curative resection of the primary tumour and before manifestation of metastasis may change our understanding of systemic cancer spread. However, in absence of manifest metastases DTCs and CTCs are extremely rare, detected only in a subgroup of patients at a frequency of 1 cell in millions of bone marrow or blood cells. We therefore developed methods to characterize the genome and the transcriptome of single cells at high resolution. The data demonstrate that cancer spread is an early event in tumour progression, that therapy targets can be identified that would be missed by analysis of primary tumours, and suggest that there is urgent need to develop a novel diagnostic pathology that directly characterizes minimal residual cancer for patient stratification and therapy selection in the age of molecular targeted therapies.

JS-3: Cell Function and Ageing

JS-3-26
Prospective isolation of self-renewing progenitors of mesodermal tissues

Bianco P.[1]
[1]*Sapienza Universita' di Roma, Italy*

Postnatal connective tissues are thought to comprise a population of multipotent cells, widely known as mesenchymal stem cells. This notion originates from the recognition that the stroma aof the post-natal bone marrow includes a population of multipotent progenitors of skeletal tissues, which can be assayed in vivo. Evidence is now accumulating pointing to subendothelial cells the specific compartment of bone marrow stromal cells that represents the in vivo counterpart of the cells explanted as clonogenic progenitors. Of not, a single surface marker, MCAM/CD146 has been instrumental in identifying both the in vivo identity of the long-sought skeletal progenitors, and of their capacity for in vivo self-renewal the defining and hitherto missing piece of evidence qualifying multipotent stromal progenitors as bona fide stem cells. The picture is becoming more complex and intriguing as additional evidence accumulates to point to a widespread class of clonogenic progenitors in a variety of post-natal mesodermal tissues, with homology in anatomy, location and phenotype, but with distinctly different potency when rigorously assayed. Thus, a novel paradigm emerges that moves the field beyond the "mesenchymal stem cell" paradigm that has dominated the last 10 years.

JS-3-27
Analysis of telomere length by in situ hybridization and flow cytometry (flow-FISH)

Baerlocher G. M.[1]
[1]*Hematology, University Hospital/Inselspital Bern, Switzerland*

Telomere length is linked to cell senescence, aging and tumorigenesis. The measurement of telomere repeats can indirectly estimate the mitotic history of cells or their residual replicative lifespan and telomere length kinetics can be of value as a molecular marker of tumor cells and cancer progression. Telomere lengths and their kinetics, however, vary among different somatic cells within an individual and these differences can be relatively small. Therefore, a highly accurate and sensitive method to measure telomere repeats in different subsets of cells within one sample is desired. We developed and further improved the flow-FISH methodology in order to measure the telomere length in granulocytes, naïve T-cells, memory T-cells, B-cells and natural killer (NK)/NKT-cells within a blood sample with high sensitivity and reproducibility (Baerlocher et al, Nat Protoc. 2006; Baerlocher et al, Methods Cell Biol. 2004). We combined flow-FISH with antibody-staining (multicolor flow-FISH) and we automated most steps in the staining protocol using a 96-well microsdispenser device (automated multicolor flow-FISH). The minimum detectable difference in telomere length and the reproducibility of the method are in the range of 0.2-0.5 kb and measurements can be made with as few as thousand cells (Baerlocher et al, Cytometry 2002 and 2003). With this improved methodology we could establish telomere length measurements by automated multicolor flow-FISH as screening test for the identification of individuals and patients with inherited defects in telomere maintenance (Alter B, Baerlocher GM et al, Blood 2007). Such testing was furthermore the basis to detect and describe new mutations in telomere associated molecules (Savage SA et al, Am J Hum Genet. 2008). In addition, we could identify very short telomeres and high telomerase activity in patients with certain types of leukemias, which seems to be an optimal situation for the treatment with telomerase inhibitors (Roeth A et al, Leukemia 2008).

JS-3-28

Dissecting the cell cycle: Loss of genomic integrity and escape from senescence

Smith P.J.[1], Errington R.J.[2]
[1]Cardiff University, Pathology, Cardiff, United Kingdom, [2]Cardiff University, Medical Biochemistry, Cardiff, United Kingdom

Chromosomal instability (CIN) is a fundamental driver for tumour progression with evidence that an initital tetraploidisation step may contribute to origins for CIN. The presentation will describe mitotic bypass as an occult route to chromosomal instability in cancer and the potential for endocycle entry to provide a means of initial escape from senescence. Experimentally we have focused on methods to dissect the cell cycle and cell lineages. It is recognized that unresolved, catenated DNA replication products can compromise mitosis but are normally sensed and resolved at a pre-mitotic 'decatenation' checkpoint (DC) in G2 by DNA topoisomerase II. We have studied the action of a catalytic inhibitor of decatenation (ICRF-193) on primary, virally transformed and tumour cells. The results suggest that ICRF-193-induced creation or persistence of chromatid anomalies, capable of generating polyploidy-CIN, can occur under circumstances that have not fully triggered a pre-mitotic checkpoint block to polyploid progression via mitosis. Thus, long-term decatenation inhibition permits limited CIN development in primary fibroblasts and spontaneously- or EBV-transformed normal lymphoblasts. CIN progression appears to be further enhanced in transformed ATM-/- lymphoblasts - consistent with a role for ATM in ICRF-193 induced CIN responses. Human p53-functional U-2 OS osteosarcoma cells were found to be capable of surviving exposure to a catalytic inhibitor and progress to a generation of aneuploid/CIN DNA profiles. Using a cyclin B1-eGFP reporter we found that ICRF-193-treated DC-proficient p53-functional tumour cells favour a transition to tetraploidy-CIN that bypasses mitosis and the mitotic spindle checkpoint. DC-deficient cells move exclusively through mitosis to tetraploidy-CIN via an endocycle. Mitotic bypass transition phase disconnects cyclin B1 degradation from nuclear envelope breakdown and allows cells to evade the action of a mitosis-targeting anticancer drugs. Bypass occurs in a background of chromatin damage and p53/ATM stress signals leading to a post-bypass elevation of nuclear p21CDKN1A and an arrest in G1 tetraploidy that can be misconstrued as an extended G2 arrest not dissimilar to that observed in genomic stress-related G2 exit in senescent cells. G1 tetrapolidy arrest is alleviated by SB203580, a highly selective and cell permeable inhibitor of p38 mitogen-activated protein kinase that can also reduce the rate of cell cycle exit in senescing fibroblast cultures. We propose that bypass could facilitate the covert development of tetraploidy/CIN in early p53 functional cancers and may offer a means by which senescent cells can escape into cycle.

(Study supported by UK MRC and BBSRC grants)

Keynote lecture

Keynote Lecture-32

Antibodies and B cell memory in immunity and immunopathology

Radbruch A.[1], Plasma Cell Group Berlin
[1]*Deutsches Rheuma-Forschungszentrum (DRFZ), Berlin, Germany*

In humoral memory, antibodies secreted into serum and other body fluids protect against repeated challenges of previously encountered pathogens. Antibody-secreting plasmacells are mostly considered to be shortlived, terminally differentiated B lymphocytes. However, in secondary lymphoid organs and in the bone marrow, plasma cells can survive for months and years. The lifetime of these long-lived plasmacells depends on an intrinsic competence to survive in a specific survival niche of those organs. The niche provides survival signals like IL-6, CXCL12 and TNF. Within a functional niche, the lifetime of a plasma cell is apparently not limited intrinsically. The number of niches in the body has to be limited, in order to maintain physiological concentrations of serum immunoglobulins. Thus recruitment of new plasmacells to the pool of old memory plasma cells has to be competetive. This competition is probably controlled by a simple molecular mechanism, namely the dual functionality of chemokines like CXCL12, which attract newly generated plasmablasts to a survival niche and at the same time are a survival signal for the plasma cell. Plasmablasts and plasma cells express CXCR4, the receptor for CXCL12. While plasmablasts migrate in response to CXCL12, plasma cells depend on it for survival in the niche, but are no longer migratory. Thus once disloged from their niche, they will die. Plasmablasts newly generated upon systemic secondary immunization, upon concommittant stimulation with interferon-gamma, can also express CXCR3, the receptor for the interferon-gamma-induced chemokines CXCL9, 10 and 11, which may lure the plasmablasts into inflamed tissue. The switch in the potential to migrate provides also an efficient means to eliminate plasma cells of the peak of an immune response, which as plasmablasts had migrated to the tissue inflamed in that pathogenic challenge. Inflamed tissue contains survival niches for plasma cells. In the inflamed tissue, plasma cells provide high local antibody concentrations. Upon resolution of the inflammation the plasma cells will be dislodged and die. Longlived plasma cells provide longlasting antibody titers (protective memory) and leave memory B cells a role in reactive memory, generating memory plasmacells in secondary challenges. In chronic inflammation, this mechanism can contribute to pathogenesis. Thus in the NZB/W model of lupus, longlived autoreactive plasma cells are generated early in pathogenesis, which survive in bone marrow and spleen. Later, in established disease, autoreactive plasma cells are shortlived and continuously generated. They do not compete with the longlived plasma cells, and both populations coexist as prominent populations. Interestingly, longlived plasmacells are resistant to therapeutic immunosuppression, while the generation of shortlived plasma cells is blocked. This may be the reason for the failure to cure antibody-mediated immunopathology, by conventional immunosuppression.

PS-4: Cell Therapy

PS-4-33

Adipose tissue as a reservoir of regenerative cells

Casteilla L.[1], Cousin B.[1], Planat-Benard V.[1], Laharrague P.[1], Bourin P.[2]
[1]UMR 5241 UPS/CNRS MPM, Toulouse, France, [2]EFS PM, Toulouse, France

Beside the classic role of adipose tissue in plastic and reconstructive surgery, the importance and the role of adipose tissues in energy balance have been greatly expanded to endocrine tissue. Some years ago, by analogy with mesenchymal stromal cells from bone marrow (MSC), the discovery that various phenotypes can be obtained from stroma cells of adipose tissue raises great hope in cell therapy and regenerative medicine. Indeed and as well known, such tissue can be easily harvested in adults and could represent an abundant reservoir of regenerative cells. Although MSC and ASC share common features and many antigenic markers, genomic, proteomic and functional studies revealed clear differences and that both cells are distinct. ASC can differentiate towards osteoblast, chondrocytes, skeletal muscles but are also strongly angiogenic via paracrine effects and a true endothelial potential. Moreover, other studies revealed that adipose tissue hosts rare cell subsets with features corresponding to true stem cells. Indeed, in mice we demonstrated that spontaneous cardiomyocytes differentiation can be achieved similar to the differentiation observed from very immature cells. However, Dani's group isolated human multipotent adipose derived cells, which display both self-renewal capability and multipotentiality. Now, one of the challenges will be to be able to prospectively sort these cells a priori. New results are strongly encouraging.
To go further and move toward clinical application, we set-up GMP conditions for ASC culture and gathered many pre-clinical data in order to perform a clinical trial in critical ischemia hindlimb. Altogether, these promising results are attracting increasing groups and investigators and in the future could lead to consider adipose tissue as one of the referent tissues for regenerative medicine.

PS-4-34

Self-renewal in ocular epithelial cells

Pellegrini G[1], Fulvio Mavilio, Michele De Luca
Center for Regenerative Medicine, University of Modena and Reggio Emilia, Modena, Italy

Purpose: Stem cells have the unique capacity to self-renew and generate committed, transit amplifying (TA) progenitors that differentiate into the cell lineages of the tissue of origin. We have recently shown that, in the human corneal epithelium, high levels of DNp63alpha identify limbal stem cells both in vivo and in vitro, whilst DNp63beta and DNp63gamma correlate with corneal regeneration and differentiation In mammary gland epithelial cells, the CCAAT enhancer binding d (C/EBPdeltą transcription factor regulates cell cycle by inducing a G0/G1arrest. The purpose of this study is to establish the molecular signatures of the self-renewing/differentiating epithelial cells.
Method: Experiments were performed on 4 uninjured and 5 wounded ocular surfaces, referred to as resting and activated, respectively. Immunofluorescence analyses were performed on ocular sections and on cultured stem cells versus differentiated cells in vitro. Forced expression of a hormone-inducible ER-C/EBPdelta chimera in human primary ocular keratinocytes was obtained. Forced expression of C/EBPḍelta and DNp63alpha by lentiviral vector was perfomed on epithelial clones.
Results: C/EBPdeltą and DNp63alfa are co-expressed by human epithelial stem cells in vivo and in vitro, and the expression of C/EBPdelta is restricted to a subset of mitotically quiescenṭDNp63alpha+/Bmi1+ cells. Forced expression of a hormone-inducible ER-C/EBPdelta chimera shows that C/EBPdelta is instrumental in regulating self-renewal and cell cycle length of epithelial stem cells. Upon injury, a fraction of these cells switches off C/EBPdelta and Bmi1, proliferates and differentiates into mature epithelial cells.

Expression of a conditional C/EBPdelta mutant inhibits the growth of epithelial colonies and increases the cell cycle length of primary epithelial cells, through the activation of p27Kip1 and p57Kip2.
Conclusion: These effects are reversible, do not alter the epithelial cell proliferative capacity, and are not due to apoptosis, senescence or differentiation. Instead, ectopic C/EBPdelta, but not DNp63alpha promotes holoclone self-renewal, as it prevents clonal evolution, suggesting that self-renewal and proliferation are distinct albeit related processes in epithelial stem cells.

PS-4-35

Potential applications of dendritic cells

de Vries I.J.M.[1]
[1]*Nijmegen Centre for Molecular Life Sciences (NCMLS), Radboud University Nijmegen Medical Centre, Nijmegen, Netherlands*

Dendritic cells (DCs) are the professional antigen-presenting cells of the immune system. Following infection or inflammation they undergo a complex process of maturation, and migrate to lymph nodes where they present antigens to T cells. Their decisive role in inducing immunity formed the rationale for DC immunotherapy: DCs loaded with tumor antigens are injected into cancer patients to stimulate T cells to eradicate tumors. Effective immune responses and favourable clinical outcomes have indeed been observed, but only in a minority of patients.
For effective immunotherapy DCs need to traffic throughout the vascular and lymphatic system to reach the T-cells located within lymph nodes. Though most immunotherapeutic agents are administered intravenously, DCs are predominantly administered intradermally. We observed that <5% of intradermally administered mature DCs reach the draining lymph nodes amounting to inefficient homing. Despite this low number we could measure effective immune responses in some patients, but generally this may be too low. We demonstrated that DC maturation is a prerequisite to exert their immunostimulatory capacity in vivo. Immuno-monitoring revealed a remarkable difference in immune responses.
In patients vaccinated with immature DC the KLH responses as well as DTH reactivity against KLH and tumor-peptides were weak and absent, respectively. In contrast, in patients vaccinated with mature DC a pronounced T cell as well a B cell response (IgG) against KLH were observed. Analysis of the response against the tumor peptides demonstrated little or no reactivity in blood. However, following intradermal administration of a delayed type hypersensitivity (DTH) challenge with gp100- and tyrosinase-peptide loaded DC essentially all patients vaccinated with mature DC showed a positive induration. Moreover, we showed the predictive value of the presence or absence of antigen-specific T cells in biopsies from DTH sites. In clinically responding patients, T cells specific for the antigen preferentially accumulated in the DTH site in accordance with the applied antigen in the DTH challenge.

PS-5: Leukemia / Lymphoma

PS-5-36

Acute myeloid stem cell analysis

Schuurhuis G.J.[1], Terwijn M.[1], Moshaver B.[1], Janssen J.J.[1], Ossenkoppele G.J.[1]
[1]*VU University Medical Center, Haematology, Amsterdam, Netherlands*

Chemotherapeutic treatment of acute myeloid leukemia (AML) patients results in high percentages (up to 80%) of complete remission. Unfortunately, half of these remission patients develop recurrence of disease, inevitably leading to death. Re-growth of disease is thought to emerge from minimal residual disease cells (MRD, typically <0.01 % - 5% of bone marrow white blood cells). MRD cells may represent cells grown out of stem cells (leukemia initiating cells), present at very low frequencies at diagnosis and resistant to therapy due to cellular resistance (including multidrug resistance and apoptosis resistance) and out of cycle characteristics. AML stem cells therefore are key in initiation, maintenance and outgrowth of disease. Proper identification and characterization of AML stem cells thus seems of utmost importance to understand the nature of the disease and to find therapeutic solutions. However, one major problem for proper identification of AML stem cells is their similarity with normal hematopoietic stem cells: both have the CD34+CD38- immunophenotype. Their frequencies (% of blast cells) usually are <1% at diagnosis AML. Several cell surface markers allow to discriminate AML from normal stem cells in the majority of AML cases (van Rhenen Leukemia 2007, Blood 2007). More recently we have found that AML and normal hematopoietic stem cells present in the same sample often differ in size and granularity (normal stem cells have lower FSC and SSC). In addition, differences in CD34 and CD45 expression on several occasions allowing to more precisely discriminate AML from normal stem cells (Monique Terwijn, unpublished). Analysis of follow up bone marrow samples reveals that both types of stem cells can be identified too in remission. Remarkably similar results as to marker expression, FSC/SSC differences and CD34/CD45 expression differences were found for chronic myeloid leukemia (Jeroen Janssen, unpublished). Lastly, in AML cases with a CD34+CD38- stem cell compartment missing, the alternative stem cell compartment is defined by Hoechst staining and referred to as the side population (SP). This compartment behaves remarkably similar to the CD34+CD38- compartment. (Moshaver, unpublished). AML and normal stem cell frequencies at diagnosis and in remission may range from 1% down to <0.001% and thereby requires high demands on adequate bone marrow sampling, instrumental set-up and gating strategies. The results now allow:
1. to isolate and molecularly and functionally characterize both AML and normal stem cells at diagnosis with the aim to design new therapeutic targets;
2. to trace AML and normal stem cells at follow up; this allows quantification of both stem cell compartments to establish the prognostic impact of AML stem cell. This “stem cell-MRD” might further be used to refine the current prospective MRD studies that aim at implementing MRD assessment as a prognostic and monitoring factor in future clinical studies.

PS-5-37

The life and death of chronic lymphocytic leukemia B cells

Damle R.N.[1], Rai K.R.[2], Chiorazzi N.[1]
[1]*Feinstein Institute for Medical Research, Experimental Immunology, Manhasset, New York, United States,*
[2]*Hematology/Oncology - Long Island Jewish Medical Center, New Hyde Park, New York, United States*

Chronic lymphocytic leukemia (CLL), characterized by the clonal accumulation of CD5 expressing B cells, is the most frequently diagnosed adult leukemia in the western hemisphere. On microscopic examination, CLL cells are uniformly small cells with a high nucleus to cytoplasm ratio, resembling resting B cells. However several cellular (surface expression of CD38, ZAP-70, CD49d, etc.) and molecular analyses (somatic mutation of Ig V genes in certain cases) have suggested at least two distinct disease sub-entities--one in

which the disease is rather stable, and the other in which the disease takes a more aggressive course and impacts both requirement of repeated treatment and patient survival. Allocation of cases to either subgroup based on the degree of somatic mutation of Ig V genes has assumed significant prognostic potential in CLL. Clonal cells from ~50% CLL cases express mutated Ig V genes (M-CLL), whereas in other patients' B cells the IgVH genes are unmutated (U-CLL). Detailed flow-cytometric analyses of clonal cells from U-CLL and M-CLL cases specifically aimed at studying markers indicative of cellular activation, adhesion, and differentiation suggest that CLL cells express features of activated, antigen-experienced cells, irrespective of their IgV gene mutation status. Furthermore, recent studies have also identified that CD38 labels a subset of the clone, enriched in cells that have traversed the cell cycle. Findings from our in vivo labeling experiments also support that CD38+ members of the clone are more proliferative than CD38- counterparts. Although the precise role of the clonal B-cell receptor (BCR) expressed by leukemic cells is not well understood, several studies imply its importance in the pathophysiology of this disease. Our studies on the replicative history of clonal B cells from these subgroups revealed shorter mean telomere lengths in U-CLL than in M-CLL, although telomerase activity was elevated in the U-CLL group. Based on the knowledge of the unmutated nature of Ig V genes of these cases and on the fact that T cell independent (T-I) stimulation of B cells drives them to respond without accumulating somatic mutations, we have assessed the effect of in vitro stimulation of purified B-CLL cells with an immunological reagent that mimics a physiological T-I trigger to B cells. We observed significantly elevated proliferative and anti-apoptotic responses to T-I stimulation by U-CLL cells but not by M-CLL cells. Importantly, the T-I stimulus also triggered/retained telomerase activity only in the U-CLL cases and not in the M-CLL cases. Repeated T-I stimulation of cells in vivo is a plausible mechanism influencing the balance between life and death of the clonal cells, at least in the U-CLL cases, and may impact the aggressive behavior of the clonal cells in this subgroup of CLL cases.

PS-5-38

Flow cytometric detection of leukemic cells in cerebrospinal fluid

Kraan J.[1]
[1]*Department of Internal Oncology, Erasmus MC, Rotterdam, The Netherlands*

Leptomeningeal disease is considered as an important adverse complication occurring in patients with B and T-cell lymphomas as well as in patients with acute leukemias of lymphoid and myeloid origin. Cytological assessment of spinal fluid samples is essential for the diagnosis of cerebrospinal fluid involvement, particularly among patients suffering from myeloid and B-cell neoplasias. Although a positive conventional cytological analysis of a spinal fluid sample is highly specific for CNS involvement, a relatively high rate of false negative results (up to 60% of the cases) has been associated with this technique. Recent reports suggest that multiparameter flow cytometry immunophenotypic assessment of spinal fluid samples could improve the efficiency of detection of CNS involvement, due to its high specificity and greater sensitivity. However, spinal fluid samples are frequently paucicellular and contain a limited number of cells with a rapidly decreasing viability. Therefore, conventional immunophenotypic protocols applied to peripheral blood, bone marrow and lymphoid tissue samples are suboptimal. Staining of spinal fluid requires dedicated sample storage/transport, staining and preparation protocols.
This presentation will review the results of diagnostic evaluations of all CSF samples analysed for localization of a hematological malignancy between 2001 and 2004 at our center and outlines a consensus protocol for flow cytometric detection published in Current Protocols in Cytometry (Unit 6.25, july 2008) this year by an international working group.

PS-6: Platelet Analysis

PS-6-39

Phenotypic and functional characterization of platelets

Alberio L.[1]
[1]*Department of Haematology and Central Haematology Laboratory, University Hospital Inselspital, Bern, Switzerland*

Platelets play a complex role in physiologic haemostasis. By adhering to exposed subendothelial structures and by aggregating, they create a physical barrier that limits blood loss. In addition, by exposing negatively charged phospholipid, activated platelets promote the assembly of procoagulant complexes on their surface. This eventually leads to a focal enhancement of thrombin generation and fibrin deposition at sites of vascular injury. Besides classical laboratory methods, which primarily assess platelet adhesive and aggregating properties, flow cytometry (FCM) offers the opportunity to investigate a wide spectrum of platelet characteristics. For instance, FCM allows to determine the surface density of structures, such as the fibrinogen receptor (defect in Glanzmann thrombasthenia) or the receptor for von Willebrand factor (defect in Bernard-Soulier syndrome), or to detect surface bound antibodies (present in autoimmune thrombocytopenias). FCM can also assess the percentage of young, reticulated platelets (useful for differentiating thrombocytopenias due to reduced bone marrow megakaryopoiesis from those mediated by increased peripheral destruction). FCM can measure content and secretion of alpha- and delta-granules (defect in storage pool diseases and secretion anomalies). Moreover, FMC can investigate several endpoints of platelet activation, such as a conformational change of the fibrinogen receptor, formation of platelet-leukocyte aggregates or surface expression of negatively charged phospholipids and generation of microparticles (increased in heparin-induced thrombocytopenia and defect in Scott syndrome and other anomalies of platelet procoagulant ability). During this lecture I will present our experience with platelet flow cytometry.

PS-6-40

Determination of platelet-specific autoantibodies in autoimmune thrombocytopenia

Tomer A.[1]
[1]*Ben-Gurion University, Faculty of Health Sciences, Beer-Sheva, Israel*

Autoimmune thrombocytopenia (ITP) is a disorder characterized by antibody-mediated accelerated platelet destruction. Despite its clinical importance, the diagnosis of which is one of exclusion, thus inevitably associated with potential difficulties. To facilitate the diagnosis of ITP, flow cytometric methods have been developed, suitable for testing a single sample as well as multiple samples. Two alternative methods have been developed.
The first method is for the determination of circulating autoantibodies against platelet-specific receptors. The method is based on the detection of autoantibodies reacting with microbeads coated with platelet-specific glycoproteins including CD41a (GPIIb/IIIa), CD42b (GPIb), CD61 (GPIIIa), CD41b (GPIIb), CD42a (GPIX), and CD51 (aV). Eighteen patients with clinical diagnosis of ITP were studied. Fourteen patients demonstrated autoantibodies against CD41a (mean fluorescence 320_137 vs. 7_2 in ten normals, and three patients demonstrated circulating antibodies against CD42b (323_147 vs. 10_3 in normals. One patient with clinical presentation consistent with post-transfusion purpura demonstrated antibody interaction with both CD41a complex and CD61 (GPIIIa) subunit (420_59 vs. 18_9 in normals. The specificity of the assay was high, and no cross-reactivity of the sera with other platelet receptors was observed.
The second method is for the detection of antigen-specific platelet-bound autoantibodies. The method is based on flow cytometric detection of autoantibodies bound to specific platelet receptors immobilized on microbeads, using glycoprotein-specific mouse monoclonal antibody. The antigen-specific assay for

platelet-associated antibody was performed in 62 adult patients with chronic ITP, 14 patients with thrombocytopenia of decreased production, and 60 healthy controls. The difference in fluorescence between the ITP patients and the control groups was highly significant, using a stringent non-parametric statistical analysis. A comparison of the flow cytometric assay with the radioactive immunobead assay previously reported on the same cohort of patients, showed significant correlation (R2 0.71). The overall performance of the flow cytometric assay in discriminating between ITP patients and normals was estimated by the receiver operating characteristic (ROC) plot, showing an area under the curve of 0.96 (maximal value 1.0), with standard error of 0.033. At a sensitivity cutoff of 86%, the specificity approached 100%, with 53 of the 62 ITP patients have a value above that level and all the healthy controls have a value below that level. Accordingly, a positive assay should be diagnostic of immune thrombocytopenia while a negative assay cannot rule it out.
In conclusion, it seems that the flow cytometric immunobead assay is practical, with relatively high sensitivity and specificity, and may be clinically useful for routine diagnosis and perhaps follow-up of patients with ITP.

PS-6-41

Platelet-induced angiogenesis and tumor growth

Broxterman H. J.[1], Verheul H. M.W.[2]

[1 and 2] *Vrije Universiteit Medical Center; Department of Medical Oncology, Amsterdam, The Netherlands*

Angiogenesis is required for tumor growth and metastasis. Tumors stimulate new vessel formation through the secretion of angiogenic factors, such as vascular endothelial growth factor (VEGF). Also, activation of the coagulation pathway enhances tumor growth and metastasis.
During recent years therefore studies have explored the putative role(s) of platelets in tumor angiogenesis. Megakaryocytes as well as platelets are known to synthesize and sequester VEGF, which is released upon activation at sites of vessel repair or neovascularisation. However, platelets contain also a repertoire of anti-angiogenic proteins such as thrombospondin and endostatin. *In Vivo* and *in vitro* evidence suggests that VEGF-stimulated endothelial cells promote platelet adhesion and activation and that *vice versa* that platelet granule secretion results in a pro-angiogenic profile and stimulation of endothelial proliferation.
We found that the VEGF-binding monoclonal antibody bevazicumab is taken up by platelets, in rabbits as well as in cancer patients, which neutralizes the VEGF activity upon platelet activation. Recent data from the literature suggesting that platelet proteins such as platelet factor 4 may be early biomarkers of malignancies and proteomic approaches to discover novel biomarkers will be discussed.

PS-7: HIV - Vaccines - Microbiology

PS-7-42

Cost-effective cytometric diagnosis in resource-poor settings

Janossy G.[1], Glencross D.K.[2], Barry S.M.[3], Breen R.A.M.[4], Tupitsyn N.[5]
[1]University College London, Harrow, United Kingdom, [2]Witwatersrand University, Molecular Medicine and Haematology, Johannesburg, South Africa, [3]BSMC, Lewes, United Kingdom, [4]Guys Hospital, London, United Kingdom, [5]N.Blokhin Russian Cancer Research Center, Immunohaematology, Moscow, Russian Federation

In recent years flow cytometry (FCM) has developed into a powerful research technology where the vast analytical power of polychromatic instruments is harnessed to unravel the immunophenotypes of mixed cell populations, the cyto/chemokine networks and biological regulatory processes. At the same time large diagnostic areas in medicine are being undervalued and appear to be lost for FCM by the introduction of alternative technologies that during the clinical applications demonstrate significantly lower degree of analytical power and potentially confusing results - when compared to the ease and precision of quantitative FCM. These, just to mention two, are the ELISpots (for detecting cytokine-secreting foci) and dipsticks (tentatively recommended for CD4+ counts). In reality, in many clinical conditions including the monitoring of HIV infection, anti-retroviral therapy and tuberculosis, simple FCM with 2- (or maximum 4-) colour fluorescence married to a straightforward service-oriented mentality, remains the optimal ally to help serious world-wide medical crisis-management. This trend can tentatively by referred to as "smart-FCM" (Clinical Cytometry 2008; 74B Supplement 1; Biotechnol J 2008, 3: 32-42).
Five examples of this approach are described:
(i) the simplified CD4+ T cell counting protocols in HIV-ridden countries, enriched by careful quality assessment (QA) - exemplified by the ´panleucogating´ protocol coupled with ´bead-rate counting´;
(ii) the introduction of lymphocyte activation tests to assist the economic use of HIV viral load tests as used in the long-established CD8-CD38 assay with recent simplifications;
(iii) the use of CD4/CD8 ratios to indicate HIV-exposure in newborns to replace expensive and slow molecular assays;
(iv) the use of FCM to diagnose active tuberculosis in two different formats: utilising the specific accumulation of TB-responsive CD4+ T cells in the sputum and broncho-alveolar lavage and to search for these lung-seeking CD4+ T cells in the blood, and, finally,
(v) the introduction of simplified ‘lite FCM’ for detecting minimal residual leukaemia in countries such as Brazil and Russia.
Here the aim is to identify children who respond exceptionally well to leukaemia-treatment and may require less-toxic therapeutic regimens as conceptually introduced by F. Pedrosa F, GK Rivera GK, D. Campana D et al. at the St. Jude Hospital, USA.
We believe that it is important for regulatory bodies and FCM firms to pay more attention to the clinical needs of problem-solving world-wide - in order to use FCM in the proper formats to exploit the full potential of these modern powerful technologies. The primary consideration in all of these methods is not merely the cost-saving but the increased quality and the easier training of the laboratory personnel. As a result, it can be documented that these techniques are performed in as high (if not superior) manner as currently is still accustomed in many ´developed´ countries.

PS-7-43

T cell response in HIV infection

Papagno L.[1]
[1]Laboratoire d'Immunologie Cellulaire et Tissulaire, INSERM U543 - Université Paris VI Pierre et Marie Curie
Hôpital Pitié-Salpêtrière, Paris, France

Antigen specific CD8+ T-cells are key players in the immune response against viruses. They achieve control of viral replication through a variety of effector mechanisms, which include the secretion of soluble anti-viral factors such as cytokines and chemokines and the direct killing of infected cells, involving degranulation of cytotoxic molecules. The analysis of their characteristics has become central in the immunomonitoring of patients enrolled in clinical trials aimed at boosting T-cell mediated immunity. New technological development in flow cytometry, in particular related to the number of fluorescent parameters that can be analysed at once, renders possible a detailed assessment of quantitative and qualitative features of antigen-specific T-cell populations. T-cell responses to viral infection have thus been widely described. However, it is not yet known what combination of phenotypes and functions, and what frequencies of antigen-specific T cells, provide protection against viruses such as human immunodeficiency virus (HIV). Understanding these issues is necessary for the rational design of effective T cell-based vaccines.

PS-7-44

Developing a new TB vaccine - boosting BCG with MVA85A

McShane H.[1]
[1]*Oxford University, Nuffield Department of Medicine, Oxford, United Kingdom*

There has never been a more urgent need for a new TB vaccine. The geographical overlap with the HIV epidemic and the emergence of multi and extensively drug resistant strains has meant existing control measures are failing to control the TB epidemic.
MVA85A is a recombinant modified vaccinia Ankara expressing the immunodominant antigen 85A from M.tuberculosis, and has been developed as a booster vaccine for BCG. Boosting BCG with MVA85A enhances BCG induced protection against aerosol challenge than either vaccine alone in mice, guinea pigs and non-human primates. MVA85A was the first new TB vaccine to enter clinical trials in 2002 and is currently in Phase II clinical trials in the UK, The Gambia and South Africa.
When used alone in BCG naïve subjects, MVA85A boosts pre-existing immunity induced by environmental mycobacteria and induces high levels of antigen specific T cells. When administered to subjects previously vaccinated with BCG, significantly higher levels of antigen specific T cells are seen. In subjects who are latently infected with M.tuberculosis, MVA85A is as safe and as immunogenic as it is in BCG vaccinated subjects. To date this vaccine is also safe and immunogenic in Gambian adults and infants; and South African adults, adolescents and children.
The main immunological readout in these clinical trials has been the ex-vivo interferon-gamma Elispot assay. We have now used a variety of cellular immunological assays including polychromatic flow cytometry, intracellular cytokine staining, CFSE proliferation and whole blood assays to characterise in more detail the vaccine induced immune responses. From this data, we see that MVA85A induces highly polyfunctional T cells which also proliferate.
This promising candidate vaccine will be evaluated in a proof-of-concept efficacy trial in infants in South Africa, beginning in 2009.

PS-8: Myelodysplastic Syndromes

PS-8-45

Identification of prognostic subgroups by flow cytometry in myelodysplastic syndromes

van de Loosdrecht A.A.[1], Alhan C.[1], Cali C.[1], Dräger A.M.[1], Ossenkoppele G.J.[1], Westers T.M.[1]
[1]*VU University Medical Centre, Department of Haematology, VU Institute of Cancer and Immunolgy (V-ICI), Amsterdam, Netherlands*

The WHO classification contributes to a more refined classification and prognostication of myelodysplastic syndromes (MDS). Flow cytometry (FC) may add diagnostic and prognostic criteria to discriminate RA from RCMD (+/- ringsideroblasts; (RS)) and may identify additional prognostic subgroups. A 4-colour FC procedure that recognises all differentiation stages of granulocytic and monocytic lineages in normal bone marrow (BM) has been developed in the past years which have been recently discussed within the ELNet workgroup for standardization. In patients with low and intermediate-1 risk MDS (RA, RARS, RCMD, RCMD-RS) aberrant expression of differentiation antigens in 1 or more lineages has shown prognostic implications. FC identified aberrancies in granulopoiesis and monocytopoiesis in >90% of low/int-I risk MDS. In the majority of cases abnormal relations between CD13, CD16, CD11b, CD15, CD10 and HLA-DR is prominent in the granulopoiesis. In approximately 1/3 of MDS cases striking monocytopenia is observed and in >60% abnormal surface expression of CD14, CD36 is found. In approximately 40% of MDS cases lineage infidelity antigen expression is detectable (co-expression of CD5, CD7, CD19, TdT or CD56 on CD34+ myeloid blasts). In patients with uni-lineage dysplasia, e.g. RA+/-RS and MDS-U, additional FC aberrancies are identified including lineage infidelity Ag expression on myeloid blasts in 30% of the cases. The number of flow cytometric aberrancies can be translated into a MDS flow-score. A significant increase in the MDS dysplasia flow-score among WHO subgroups from RA+/-RS, RCMD+/-RS to RAEB1/2 is a common feature. Within IPSS subgroups flow-scores are highly heterogeneous which might identify separate disease entities. Interestingly, a significant increase in the MDS dysplasia flow-score is observed between non-transfusion dependent low/int-I risk patients and patients in progression to advanced MDS. In half of the patients with transfusion-dependency and/or in progressive disease, infidelity markers on myeloid blasts are detected. In contrast, in only a minority of non-transfusion dependent patients an infidelity marker on myeloid blasts is identified. Within the pure-RA+/-RS subgroup only patients with infidelity marker expression on myeloid blasts are transfusion dependent. Furthermore, it is recognized that the MDS flow-score identifies patients with a worse clinical outcome after allogeneic stem cell transplantation. It is concluded that FC in MDS identifies aberrancies in the granulocytic and monocytic lineages and classifies patients with multilineage aberrancies not otherwise determined by cytology (WHO). FC may discriminate pure RA from FC-defined RCMD. FC identifies patients at risk for transfusion dependency and/or progression towards high risk MDS independent of known risk groups and patients with worse clinical outcome after allogeneic transplants.The exact role of the MDS dysplasia flow-score on treatment decisions and as tool in disease monitoring has to be determined in ongoing prospective studies.

PS-8-46

The molecular signature of MDS stem cells supports a stem-cell origin of 5q myelodysplastic syndromes.

Nilsson L.[1]
[1]*Hematopoietic Stem Cell Lab, Lund Stem Cell Center and Dept of Hematology, Lund University Hospital, Sweden*

Global gene expression profiling of highly purified 5q-deleted CD34+CD38–Thy1+ cells in 5q– myelodysplastic syndromes (MDSs) supported that they might originate from and outcompete normal CD34+CD38–Thy1+ hematopoietic stem cells. Few but distinct differences in gene expression distinguished

MDS and normal stem cells. Expression of BMI1, encoding a critical regulator of self-renewal, was up-regulated in 5q– stem cells. Whereas multiple previous MDS genetic screens failed to identify altered expression of the gene encoding the myeloid transcription factor CEBPA, stage-specific and extensive down-regulation of CEBPA was specifically observed in MDS progenitors. These studies establish the importance of molecular characterization of distinct stages of cancer stem and progenitor cells to enhance the resolution of stage-specific dysregulated gene expression.

PS-9 Interactive voting – controversies

PS-9-48

Low abnormal cell numbers in chronic lymphoproliferative disorders: they are really important!

Rawstron A. C.[1]
[1]*Department of Haematology, St. James's Institute of Oncology, Leeds; UK*

Minimal levels of phenotypically abnormal cells are important in two settings: i) detection of minimal residual disease (MRD) after treatment for progressive disease and ii) analysis of abnormal cells in otherwise healthy individuals.

As more effective treatments are developed, so more sensitive assays are required to assess response and compare different treatment approaches. In B-cell chronic lymphocytic leukaemia (B-CLL), the overall response rate has increased from less than 10% to more than 70% over the past few decades, and sensitive methods to detect MRD are now a requirement for response assessment in clinical trials. There is good correlation between the level of residual disease and both the progression-free and overall survival in most chemo-immunotherapy trials. There are studies demonstrating that depletion of MRD improves survival but randomised trials are difficult to design and this remains a controversial area. The relevance of MRD is also less clear after allogeneic transplantation. However MRD assessment is becoming standardised and routinely applicable in many settings.

The improved sensitivity or routine diagnostics has led to the detection of apparently abnormal cells in large proportions (5-10%) of otherwise healthy individuals. In CLL this has been particularly problematic as the 1996 diagnostic criteria were open to interpretation, allowing people with minimal levels of abnormal cells to be classified as having leukaemia, potentially causing psychological and financial difficulties. The alternative approach is to disregard very low levels of abnormal cells because so few affected individuals will show disease progression. However, the abnormal cells present in the general population are similar to those in patients with leukaemia not only in terms of phenotype, they can also demonstrate molecular abnormalities that are characteristic of progressive disease. Approximately 1% progress of individuals presenting with a low level of CLL-phenotype cells will develop progressive CLL requiring treatment per year of follow-up. Monitoring can pre-empt severe infectious episodes or progressive anaemia but it may be unnecessary for many otherwise healthy people, or invasive for people with significant unrelated health issues. Investigation of the biological features of the abnormal cells helps to identify which individuals will benefit from monitoring, and also provides an insight into the mechanisms by which haematological cancers initiate and transform.

PS-9-50

Flow Cytometry provides clinically important information

Orfao A.[1]
[1]*Cancer Research Center (IBMCC-USAL/CSIC), Department of Medicine and Cytometry Service, University and University Hospital of Salamanca, Salamanca, Spain*

Multiparameter flow cytometry immunophenotyping has recurrently proven to be of clinical utility in the differential diagnosis of monoclonal gammopathies, evaluation of the probability of progression of smoldering multiple myeloma (SMM) to symptomatic myeloma, and monitoring of minimal residual disease levels after intensive therapy. Accordingly, it has been shown that an increased ratio (> 95%/5%) between the percentage of (mono)clonal myeloma plasma cells (PC) and normal polyclonal PC within the overall bone marrow (BM) PC compartment would be highly suggestive of multiple myeloma (or evolution towards a symptomatic multiple myeloma within SMM), while a lower ratio would support the diagnosis of monoclonal gammopathy of undetermined significance (MGUS) or smoldering, non-progressing myeloma. In fact the aberrant/normal PC ratio has been shown to be the most powerful single parameter for the discrimination between MGUS vs MM and between stable vs progressing SMM. In turn, clearance of phenotypically aberrant myeloma PC after therapy is associated with a better stringent complete remission and patient outcome. In contrast, expression of individual markers on clonal PC does not appear to have a significant impact on patient survival, when tested in large series of patients receiving high dose chemotherapy followed or not by an autologous stem cell transplant.
Despite all the above, the use of multiparameter flow cytometry immunophenotyping in myeloma patients is still restricted in many centres to the differential diagnosis of a few infrequent cases. Such discrepancy between the utility of multiparameter flow cytometry and the extent of its use in routine diagnosis is mainly due to a few early observations and technical questions, but no real scientific reasons. Firstly, for many years there were no PC markers available for the specific identification of BM PC. Secondly, early studies showed the existence of discrepant results between the histopathological/cytological and immunophenotypical BM PC counts, suggesting the later were not reliable in a significant proportion of cases. In addition, a reproducible, easy discrimination between normal and clonally aberrant BM PC requires simultaneous assessment of >3 individual markers, which only became standard practice in many laboratories from the late nineties on, and different results are frequently obtained with different combinations of fluorochrome conjugated monoclonal antibody reagents (e.g.: CD38FITC - CD56PE - CD45PerCP - CD138APC - CD19PC7 vs CD56FITC - CD38PE - CD138PerCP - CD45APC - CD19PC7. Recent consensus recommendations of the European Myeloma Net (EMN) provide guidance to apply multiparameter flow cytometry immunophenotyping in the diagnosis and monitoring of myeloma patients to be easily implemented and widely applied.

PS-9-51

Pre-clinical assessment of multiple myeloma – a case against flow cytometry

Rahemtulla A.[1]
[1]*Department of Haematology, Imperial College, Hammersmith Hospital, Du Cane Road, London W12 0NN*

Multiple myeloma (MM) is a clonal B-cell disease characterized by the accumulation of abnormal plasma cells, bone destruction, renal dysfunction and immunodeficiency. The median age at diagnosis is 65 years, and the median survival with conventional chemotherapy is about 3 years. MM is responsible for about 1% of all cancer-related deaths in Western Countries. The diagnosis of MM is established by the demonstration of the abnormal protein (paraprotein) in the serum and/or the urine, the presence of more than 10% plasma cells in the bone marrow, low levels of normal antibodies, bone lesions, or plasmacytoma.
Assessment of myeloma includes measuring haematological, biochemical and radiological parameters. Cytogenetic abnormalites have been described in myeloma but the precise significance of these abnormalities remains unclear and prospective studies are underway to investigate this. Flow cytometry

may help in making an earlier diagnosis but this information may not necessarily help in improving outcome from earlier treatment. Signs of disease progression, such as rising paraprotein levels, falling haemoglobin levels, radiological evidence of bone disease, or an increase in bone marrow infiltration in non-secretory myeloma, are all indications to start treatment. High dose therapy and autologous stem cell transplant (ASCT) is now considered standard treatment for myeloma in eligible patients after chemotherapy. Good responses with complete remission rates up to 30% can be achieved after ASCT, even in patients refractory to conventional treatment, leading to longer progression free survival and overall survival. Some patients in complete remission may have minimal residual disease as detected by polymerase chain reaction (PCR) or flow cytometry. The significance of this will be discussed during the debate and arguments will be presented to convince the audience that although flow cytometry may play an important part in the management of myeloma in the future, it is of limited value at present.

Poster Session

P-1: Cytometry in Microbiology and Biotechnology

P-1-126

A genome size analysis in hamsters of different species and hamster cell lines

Ryazanova M.S.[1], Kudryavtsev B.N.[1], Rozanov Y.M.[2], Sakuta G.A.[1]
[1]*Institute of Cytology Russian Academy of Science, Cell Pathology, Saint Petersburg, Russian Federation,*
[2]*Institute of Cytology Russian Academy of Science, Chromosome stability, Saint Petersburg, Russian Federation*

The goal of the work was to study variations of genomic DNA content in hamsters of different species and immortalized hamster cell lines. Spleenocytes of four hamster species Cricetulus griseus, Cricetulus barabensis, Cricetulus pseudogrizeus, Phodopus sungorus and two cell lines derived from Chinese hamster (Cricetulus griseus) V-79 (the lung, 2n=22, modal number 21) and CHO-K1 (the ovary, 2n=22, modal number 20) were stained with ethydium bromide and a flow cytometry was used for nuclear DNA content determination. DNA contents were 6.63, 6.43, 6.71 and 5.70 pg per nucleus for Cricetulus griseus (2n=22), Cricetulus barabensis (2n=20), Cricetulus pseudogrizeus (2n=24) and Phodopus sungorus (2n=28) respectively. These data is a strong evidence of Phodopus sungorus belonging to a separate genus due to a significant lower genomic DNA content ($P < 0.001$). When cells live in cultures or at transformations the DNA content can be changed. Genomic DNA contents in V 79 and CHO-K1 cell lines were 6.38 and 6.53 pg ($P < 0.01$ vs. spleenocytes of Cricetulus griseus) respectively. Taken together, presented data indicate that genomic DNA size analysis can be applied for taxonomic and cell transformation studies.

P-1-127

Additional DNA in human cell line genome

Sakuta G.A.[1], Agafonova N.A.[1], Baidyuk E.V.[1], Bezborodkina N.N.[1], Rozanov Y.M.[2], Kudryavtsev B.N.[1]
[1]*Institute of Cytology Russian Academy of Sciences, Cell Pathology, Saint Petersburg, Russian Federation,*
[2]*Institute of Cytology Russian Academy of Sciences, Chromosome stability, Saint Petersburg, Russian Federation*

Flow cytometry was used to study the nuclear DNA contents in 40 human cell lines. Cells were stained with ethidium bromide and a flow cytometry was used for nuclear DNA content determination. The analysis of DNA content has shown the presence of an additional genetic material in genome of all cell cultures. Degree of aneuploidy in various cell lines varied from 0.12 to 12.44%. The image microfluorimetric method was used for measure DNA content in individual chromosomes of CCRF-SB (peripheral blood, acute lymphoblastic leukemia) cell line karyotype. The microfluorimetric method includes traditional preparation of metaphase chromosomes. Then after identification of Hoechst 33258 - stained chromosomes, the dye was removed and the chromosomes were stained by Feulgen, using the Schiff-type reagent Auramine 00-SO_2. The measurement of DNA content in individual chromosomes was performed using an image analyzer. It was shown that the differences of DNA content per chromosome can be reached 20% comparing control (analogous chromosomes of health human). The presented data provide evidences that destabilization of cell genetic apparatus in cell culture can be found on genomic and chromosomal levels.

The work was supported by Grant of Saint Petersburg City Government.

P-1-128

Monitoring the functionality of yeast cells using flow cytometry

Sommer S.[1], Hutter K.-J.[2]
[1]DLR Rheinpfalz, Weinbau und Oenologie, Neustadt, Germany, [2]FH Mannheim, Biotechnologie, Mannheim, Germany

The aim of this study was to identify parameters and to establish methods for viable yeast cells that help to assess the functionality of the metabolism and the vitality of the cell. Throughout the fermentation process yeast is facing changing conditions and has to react by adapting its metabolism. The adaptation process is the critical point in every fermentation and is based on the accurate function of the cell. The membrane-potential, which is, for example, responsible for protecting the yeast from low pH-values, plays an important role for evaluating functionality. Other direct indicators discussed in this work are storage products like glycogen and neutral lipids, as well as mitochondrial activity and the degree of DNA damage. These parameters are completed by the measurement of indirect products and the closer inspection of following reactions of the yeast cell like viability, cell cycle, intra-cellular pH-value and reactive oxygen species inside the cell. The correlation of these factors provides useful criteria for evaluating the performance and refractiveness of the yeast. In the present test-series a Saccharomyces cerevisiae yeast was conducted in a must media for 384 hours (16 days) and analysed for its viability and functionality parameters. The results show a clear confirmation of theoretical coherence. Furthermore it is possible to get a deeper insight in stress response and adaptation behaviour of yeast. The causality from the formation of oxygen-radicals to necrosis or apoptosis could be proved metrologically, as well as the dependency of the intra-cellular pH-value on the stability of the membrane. This bio-monitoring system using Flow Cytometry provides valuable information as a time-saving process control for wineries.

P-1-129

Monitoring fermentation performance and stress response of wine yeast (Saccharomyces cerevisiae) using flow cytometry

Sommer S.[1], Schuster C.I.[2], Hutter K.-J.[3]
[1]DLR Rheinpfalz, Weinbau und Oenologie, Neustadt, Germany, [2]FH Geisenheim, Wiesbaden, Germany, [3]FH Mannheim, Biotechnologie, Mannheim, Germany

The aim of this study was to establish new methods to control wine and beer fermentation with the help of direct parameters. In wineries and breweries fermentation performance is usually measured by analysing sugar uptake and alcohol increase. The viability parameters and nutrient situation inside the yeast cell is no monitored, which is often a problem when it comes to stuck fermentation. Measuring storage products and autolysis enzyme activity inside the cell, fermentation problems can be recognized before yeast metabolism stops working.
A procedure was developed to stain glycogen in yeast cells to detect this molecule using flow cytometry. The specific staining was carried out with acriflavine according to the procedure of Gharton et al. and Meyer et al. with minor modifications. Cell cycle, trehalose and neutral lipids were measured according to Hutter et al. (1978 - 2003). The Autolysis activity assay was carried out with the EnzChek Protease Assay Kit®. Two yeast strains were used for these experiments. The wine yeast G74 from Geisenheim was inocculated in must and synthetic worth. The bottom fermenting beer yeast was supplied by the Eichbaum Brewery Group Mannheim. Fermentations were conducted for 70 and 180 hours to analyse the storage substances and for 180 hours to look at the physiological state during fermentation. To simulate controlled stress situations for the yeast some fermentation experiments were conducted at 4°C, 40°C and at pH 2.8. The results show some very interesting stress responses. Especially trehalose and neutral lipids seem to play an important role in the cell protection system. This bio-monitoring system using Flow Cytometry provides valuable information as a time-saving process control for wineries.

P-1-130

Investigation of glycogen fractions content in rat hepatocytes using Schiff's reagents (auramine-SO2 and ethidiumbromide-SO2)

Mushinskaya E.V.[1], Yakupova G.S.[1], Bezborodkina N.N.[1], Kudryavtsev B.N.[1]
[1]*Institute of Cytology Russian Academy of Science, Laboratory of Cellular Pathology, Saint Petersburg, Russian Federation*

At the present time data about glycogen structure in hepatocytes now it is possible to receive only in the form of the average values describing a cellular population as a whole. Existing approaches do not allow defining the easily soluble and hardly soluble glycogen fractions or their composition in separate cells and search of methods which permit to investigate the glycogen structure in separate cells represents an actual problem. Dynamics of glycogen and of its fractions contents were determined in hepatocytes after glucose administration to fasting rats. The content of glycogen in hepatocytes was determined in preparations-smears of isolated cells. Different glycogen fractions in hepatocytes were detected using a fluorescent PAS-reaction (with Schiff's reagents with various spectral characteristics - auramine-SO2 and ethidiumbromide-SO2) has allowed revealing two glycogen fractions - easily soluble fraction (ES, 40 min of coloring) and hardly soluble fraction (HS) that completely colored only within 90 minutes. For measurement of fluorescence intensity of hepatocytes a Videotest image analyzer (Ista-Videotest, Ltd., St. Petersburg) consisting of a luminescent microscope ES LUMAM RPO 11, black-and-white CCD-camera and IBM PC a compatible computer.
The obtained data indicate that glucose load stimulated of glycogen synthesis. As a result, as early as in 10 min the glycogen content in hepatocytes was higher than in cells of fasting animals. It was established, that with accumulation of glycogen content in cells ES-fraction grows up. Correlation coefficient (r) between content of ES-fraction and total glycogen for hepatocytes derived from liver of starving rats was 0.95, and for hepatocytes through 20 and 30 min after administration of glucose to starving rat - 0.95 and 0.98, correspondingly. In the period of sufficiently synchronous synthesis of glycogen by hepatocytes coefficient of variation of values of ES/HS ratio in cells was low (v = 13.3, 13.7 and 9.9%, for 0, 20 and 30 min after administration of glucose, correspondingly). Through 90 and 120 min after administration of glucose, when quantity of glycogen reduced in cells, dependence of ES-fraction content from total glycogen content became less pronounced (r = 0.71 and 0.82, correspondingly). Simultaneously with decrease of correlation coefficient (r) between content of ES-fraction and total glycogen in hepatocytes coefficient of variation of values ES/HS ratio in cells increased (v = 31.1 and 30.1%, correspondingly).
These results allow us to conclude, that the contents of easily soluble fraction of glycogen in hepatocytes increases, when glycogen synthesis prevails above its degradation, and is reduced, if glycogen degradations prevails above its synthesis. Therefore, accumulation of glycogen in cells occurs predominantly due to increase of ES-fraction.

The work was supported by Russian Fund of Fundamental Investigation (RFFI No. 08-04-00971-a).

P-1-131

FCM ploidy analysis as an important tool in evolutionary studies, as demonstrated by Melampodium cinereum (Heliantheae, Asteraceae)

Obermayer R.[1], Reich D.[1], Rebernig C.A.[1], Blöch C.[1], Weiss-Schneeweiss H.[1], Stuessy T.F.[1]
[1]*Institute of Botany, Systematic and Evolutionary Botany, Wien, Austria*

Originally coming from the field of medical research and diagnostics, flow cytometry (FCM) nowadays is an important tool for a great variety of applications, also in terms of clarifying questions on biodiversity. Concerning its usage in plants, its role for ploidy analysis has been strengthened due to rapid development of molecular tools and their demand for interpreting molecular results on the basis of correct ploidy data. FCM provides a rapid screening method for obtaining ploidy data of the often very extensive amount of the

investigated taxa.
The genus Melampodium comprises 39 recognized species which are mainly distributed in Mexico, with some species in Central America and the southwestern US. All species have yellow rays except for three shrubby, xerophytic taxa, M. leucanthum, M. cinereum and M. argophyllum, which form the so-called white-rayed complex and mark the northern limits of the genus distribution range. These three species are clearly separated from the rest of the genus by their ecology and form a well supported group in sequence analysis of cp and nrDNA.
The current study concentrates on ploidy distribution and biogeography in M. cinereum. It presents the data on the ploidy levels estimations for 30 populations (450 individuals) of M. cinereum, covering the varieties: M. cinereum var. cinereum (21 populations, 274 individuals), M. cinereum var. hirtellum (6 populations, 139 individuals) and M. cinereum var. ramosissimum (3 populations, 37 individuals). For ploidy determination, flow cytometry of DAPI stained silica gel-dried material has been applied, using Glycine max 'Merlin' as the internal standard.
AFLP fingerprinting analysis was conducted on 25 of these 30 populations using 6 primer combinations, to analyse the genetic structure and diversification of the three varieties and especially of the tetraploid individuals.
Eight populations of M. cinereum var. cinereum (39 individuals) are diploid and 13 populations (235 individuals in total) are 4x, (three contain sporadically 5x individuals, and one 6x individual). All 6 populations of M. cinereum var. hirtellum are diploid (one containing a 3x individual). All 3 populations of M. cinereum var. ramosissimum are diploid.
The polyploid individuals within the var. cinereum form a distinct genetic entity clearly separated from the diploid populations. The correlation of ploidy levels in M. cinereum with the molecular and cytogenetic data suggests single (or few time) origin of polyploids and their subsequent establishment in a new ecological niche.

P-1-132

Use of Qdot® nanocrystal conjugates for multispectral flow cytometry studies
Godfrey W.L.[1], Zhang Y.-Z.[1], Buller G.M.[1], Clarke S.[1], Jaron S.[1]
[1]*Invitrogen Corporation, Eugene, Oregon, United States*

Researchers trying to get more information out of their flow cytometry experiments by increasing the number of parameters observed in each sample have been limited by available fluorophores and the need for complex spectral overlap corrections between fluorophores (compensation). Qdot nanocrystals provide powerful tools to multiply fluorophore selection, particularly with the advent of direct conjugates against a range of cell surface markers. These semiconductor nanocrystals are optimally excited by ultraviolet or violet light, and provide sharp, symmetrical emission peaks that can be 150 to 400 nm above their excitation wavelengths. Unlike conventional fluorophores such as fluorescein and phycoerythrin (RPE), a Qdot nanocrystal is also excited with decreasing efficiency by wavelengths up to its emission wavelength. However, care is required with filter selection and with attention to cross-laser (rather than within-laser) compensation for spectral overlap. In this study, we review the use of Qdot nanocrystal conjugates in multicolor combinations with conventional fluors, with particular attention to filter selection to minimize the impact on fluor combinations that show severe spectral overlap with particular nanocrystals. All experiments were performed using standard staining protocols with available conjugates (Invitrogen, Carlsbad, CA) on human peripheral blood leucocytes and were analyzed using a BD LSR II flow cytometer. Where necessary, alternate long pass and band pass filters were specified based on emission spectra and tested versus default filter configurations. For example, Qdot 655 nanocrystal emission, normally detected with 405 nm excitation and a 655 nm band pass filter, was also observed in the APC channel (633 nm excitation, 660 nm band pass), but could be removed with less than 20% compensation. On the other hand, Qdot 605 nanocrystal emission in the RPE-Texas Red® dye channel (488 nm excitation, 610 nm band pass) required more than 100% compensation to be removed, but compensation could be reduced to less than 50% by using a 620 nm band pass filter while retaining adequate resolution of the populations detected with the

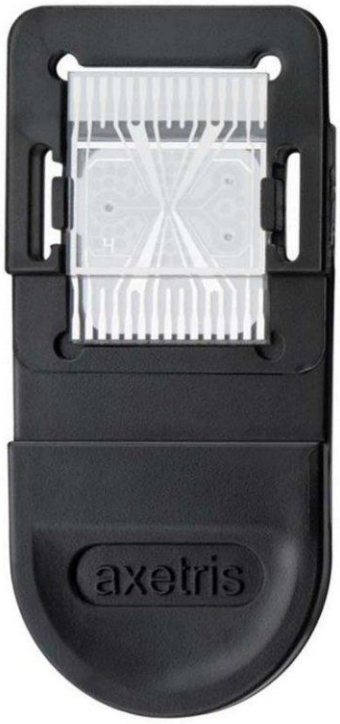
tandem dye. As a result of individual channel optimizations, 8 to 12 color experiments have been run, including 2 to 4 Qdot nanocrystal conjugates, with good resolution of cell populations. Due to the unique spectral characteristics of nanocrystals, Qdot nanocrystal conjugates can dramatically facilitate multicolor experimental design when performing flow cytometry experiments.

P-1-133

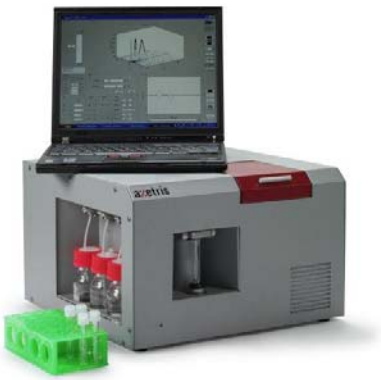
A microchip-based impedance flow cytometer for label-free, non-invasive single cell analysis

Hebeisen M.K.[1], Schade G.[1], Ziswiler A.[1], Hessler T.[1], Di Berardino M.[1]
[1]*Leister Process Technologies, Axetris Microsystems Division, Kägiswil, Switzerland*

Flow cytometry has become a routine technique for cellular analyses. However, this technology is still very complex and usually requires costly colour reagents as well as specifically trained people for its operation. In the past decade, therefore, many research activities in the field of microfluidics and lab-on-a-chip devices aimed at miniaturizing, simplifying and reducing cost of systems and consumables. Among others, many chip-based flow cytometers relying on electrical measurement techniques were developed. However, these developments had primarily academic relevance, were rather used for cell counting or size discrimination purposes and ended up by showing the proof-of-concept only. Here we provide evidence that the microfluidic chip approach is not just an academic playground, but has its entitlement as a valuable and alternative single-cell analysis tool.



[Microfluidic Chip]



[Impedance Flow Cytometer]

Based on a microfluidic chip we developed a flow cytometer prototype whose technology relies on impedance measurement. This impedance flow cytometer determines the electrical impedance of cells at up to four electrical frequencies simultaneously, providing information about volume, membrane capacitance and cytoplasm conductivity of every single cell. The obtained data allows for discrimination of cell size, metabolism, intracellular and extracellular structure, viability or cell type. Since the measurement occurs in a non-invasive and label-free manner neither extensive sample preparation nor costly cell labelling procedures are required. Moreover, the cells can even be recovered and used for downstream processes after cell analysis. Several applications in the field of cell physiology and differentiation, haematology, parasitology and microbiology were already presented. Here we will discuss in detail additional results obtained from animal and microbial cell analysis experiments, such as viability, biotechnological production and discrimination of cell types.
In summary, this microfluidic approach combines the advantages of performing assays in small sample volumes with minimal sample preparation efforts and without the need of any cell labelling. The presented studies provide a basis for addressing further potential applications in various research fields. The device, which can also be designed for cell sorting applications, represents a powerful pre-diagnostic cell analysis tool and important complement to the known cell analysis instruments. Its attributes, together with its simplicity of use particularly assign the impedance flow cytometer to quality control and near-inline routine applications.

P-1-134

Dual pulse labeling of S-phase population using click chemistry

Clarke S.T.[1], Bradford J.A.[2], Godfrey B.[2]
[1]*Molecular Probes/ Invitrogen, Universal Labeling & Detection Technologies, Eugene, United States,*
[2]*Molecular Probes/ Invitrogen, Flow Cytometry, Eugene, United States*

Multiple pulse labeling of DNA of proliferating cells based on the incorporation of nucleoside analogs is a standard method for monitoring time dependant changes in proliferation rate. Typically, halogenated derivatives of deoxyuridine are used and detected with antibodies which have selective affinity for the specific halogen modified base. Although there are several halogen modified bases to use, antibody selectivity must be carefully evaluated to achieve correct labeling. By augmenting the antibody approach with a non-antibody method of detection using a click chemistry modified deoxyuridine analog, another specific label can be added to the tools used for examining changes in proliferation rates. Here, examples are shown combining click chemistry with traditional BrdU antibody labeling in both suspension and adherent cell based models. Click chemistry labeling uses 5-ethynyl 2′-deoxyuridine (EdU) incorporation and detection with copper catalyzed dye-azide to form a stable covalent triazole ring. Use with selected bromodeoxyuridine (BrdU) antibody results in unambiguous double labeling without cross-reactivity. Fixation and permeabilization protocols which are compatible with BrdU detection are also compatible with click labeling. An unanticipated benefit is observed when using these two nucleoside analogs in combination. Demonstrated by flow cytometry in Jurkat and Tf1 suspension cells, simultaneous addition of BrdU and EdU is shown to only incorporate BrdU into the newly synthesized DNA, while sequential addition of BrdU following an EdU pulse without prior removal of the EdU results in 3 population of cells: firstly, those labeled only with EdU during the first pulse are cells which have exited S-phase prior to BrdU addition, secondly, those labeled with both BrdU and EdU are cells which have remained in S-phase during the duration of the two pulses, and thirdly, those only labeled with BrdU are cells which entered S-phase after BrdU addition. This third population (and the simultaneous addition of the two analog example) would normally be expected to be double labeled but are not. Examples of both long and short pulses of double labeling are given and the complex labeling patterns are interpreted through the use of appropriate population gating. This no wash double labeling phenomena can be used to advantage since cells just entering S-phase at the time of addition of the second label can be uniquely labeled with a single analog. Rapid changes in proliferation rate induced by drug or treatment can be monitored using this no wash pulse labeling method.

P-2: Tissue Analysis and Imaging

P-2-135

The cellular mechanisms of glycogenesis in cirrhotic liver hepatocytes after administration of glucose to starving rats

Bezborodkina N.N.[1], Vakhtina A.A.[1], Kudryavtsev B.N.[1]
[1]*Institute of Cytology Russian Academy of Sciences, Laboratory of Cellular Pathology, Saint Petersburg, Russian Federation*

The dependence between content of glycogen and cell dry mass and ploidy level in hepatocytes of rat cirrhotic liver after giving glucose to hungry animals was investigated. Cirrhosis of rat liver was induced by chronic CCl_4 poisoning during 6 months. Samples of isolated hepatocytes were prepared from rat cirrhosis liver after animals starving during 48 hours and after 10 and 60 minutes after giving glucose to animals. We measured dry mass of liver cells, content of glycogen and DNA content by using combination cytophotometrical method. This method allows measuring different parameters in one cell. Content of glycogen and DNA content were measured by using cytofluorimetry, and cell's dry mass was measured by using interferometer microscope. To determine cell ploidy, the preparation-smears of isolated hepatocytes were stained for DNA using the Schiff-type reagent auramine-SO_2. The DNA content in hepatocyte nuclei was measured using a RIF-1 impulse microfluorimeter. The mean cell ploidy was calculated by the formula $g \equiv \Sigma n_i \times 2^i$, where n_i - relative number of hepatocytes of the i-st ploidy class (i = 0 - diploid class, i = 1 - tetraploid class, etc.).
Dry mass of rat hepatocytes in the preparations-smears was measured using a MBIN-4 interference microscope (LOMO, St. Petersburg). The measurements were performed at two stages. First the optical density was determined for the cell and the medium by using glycerol as an embedding medium. Then, using an image analyzer, the cell area (in μm^2) was measured. The dry mass of hepatocytes was calculated by the formula: $P = \delta S/100\alpha$, where P - dry mass of the cell (in picograms), S - cell area (μm^2), α - relative increment of refractive index that amounts for proteins in glycerol to 0.00095 cm^3/g, δ - difference of the light beam pathway (in cm^2), which was determined by the formula: $\delta = (\varphi 1 - \varphi 2)\ \lambda/\hat{E}$, where δ - difference of the light beam pathway, $\varphi 1$, $\varphi 2$ - readings at the Senarmon□s scale, λ- the light wavelength (526 nm), $\hat{E} = 180^o$.
To the glycogen content in individual hepatocytes determined the preparations of isolated cells were stained with a fluorescent variant of the PAS reaction. Then the content of glycogen was measured by the method of cytofluorimetry, using a LUMAM IUF-3 cytofluorimeter (LOMO Co., St. Petersburg).
It was shown that dry mass and content of glycogen in hepatocytes of cirrhotic liver are proportional of ploidy level. There isn't the dependence between contents of glycogen in hepatocytes and its dry mass. And it was shown that all hepatocytes can syntheses glycogen. But different hepatocytes have different speed of glycogen hoarding. And speed of glycogen hoarding didn't depend of hepatocytes sizes.

The work was supported by Russian Fund of Fundamental Investigation (RFFI No. 08-04-00971-a).

P-2-136

Calibration of in-vivo fluorescence imaging

Mittag A.[1], Tárnok A.[2]
[1]*University of Leipzig, Translational Centre for Regenerative Medicine, Leipzig, Germany,* [2]*University of Leipzig, Dept. of Pediatric Cardiology, Leipzig, Germany*

Stoichiometric fluorescence analyses require information about fluorescence intensities and their relation to the number of fluorochromes or fluorescent cells detected. Quantitative fluorescence analysis in whole animals is still challenging. The measured fluorescence intensity depends on location and depth of the light emitting object in the animal as well as on scatter and absorption effects. Nevertheless, calibration is unavoidable to quantify fluorescence intensities. This is of particular importance in long-term follow-up

studies, e.g. in tumor treatments. Common for this purpose in cytometric analyses is calibration with fluorescent particles. These beads have different fluorescence intensities and known corresponding numbers of fluorochromes. With that knowledge it is possible not only to determine the resolution of the instrument but also to obtain a direct correlation to the number of bound fluorochrome molecules. Comparable calibration does presently not exist for in-vivo fluorescence analyses. Here we present a method which allows for evaluation of the number of fluorescent events (i.e. cells in most cases) in fluorescent spots in animals. Solutions of pure, unconjugated fluorochromes (FITC, methylene blue) with a range of 1pg/ml-500µg/ml were analyzed in multi-well plates by Maestro In-Vivo Imaging System (CRi Inc.) and by iCys Research Imaging Cytometer (CompuCyte Corp.) for comparison. Parameters for fluorescence acquisition were set for optimal detection. The detected signal in a defined area was divided by exposure time or PMT percentage in order to gain setting-independent relative fluorescence intensity values.
Both systems yield good linear relationships between dye concentration and fluorescence intensity. However, there are differences in sensitivity between these two instruments. Whereas iCys provide optimal results also in the pg range below 0.1µg dye/ml, the Maestro System shows a good linear correlation from 5ng/ml up to 30µg/ml.
Although this method does not include scatter and absorption effects it provides a basis for future calibration of fluorescence in-vivo analyses and should be used in every analysis. Both instruments, if combined, provide the opportunity for detailed fluorescence analyses in tissue. In-vivo imaging allows for detection of tissues of interest and the determination of cell numbers within a fluorescence hot spot. iCys analyses can yield subsequently cytometric data of this tissue.

P-2-137

Evaluation of optimal DNA staining for triggering by Scanning Fluorescence Microscopy (SFM)
Marecka M.[1], Mittag A.[1], Pierzchalski A.[1], Malkusch W.[2], Bocsi J.[1], Tárnok A.[1]
[1]University of Leipzig, Pediatric Cardiology, Leipzig, Germany, [2]Carl Zeiss Imaging Solution GmbH, Zeiss Group, München-Hallbergmoos, Germany

Background: DNA staining is one of the most common trigger signals for cell identification. In addition, DNA-staining is the golden standard for instrument calibration. However, selection of the proper DNA dye from the plethora of available DNA dyes is restricted by the hardware configuration of the instrument. The Zeiss Imaging Solution GmbH (Hallbergmoos, Germany) introduced a new automated scanning fluorescence microscope-SFM that combines imaging with cytometric parameters measurement. The aim of the study was to select optimal DNA dyes for triggering leukocyte detection and subsequent cytometric analysis of double-labeled leukocytes by SFM.
Methods: Axio ImagerZ1 fully motorized microscope equipped with high-resolution digital camera (AxioCam MRm) and AxioVision software (automatic multi-channel scanning and fluorescent analysis) was used. For the experiments 12 DNA dyes were tested. Three of them were found to work with the filtersets (fs) of the SFM and produce relatively low CV-values (DAPI: fs 49, POPO-3: fs 44, propidium-iodide (PI): fs 20; all dyes from Invitrogen; all fs from Zeiss). Blood mononuclear cells isolated on 1.077 density gradient were used. Cell smears were fixed and stained with DNA dyes and monoclonal antibodies labelled with Alexa Fluor 350, FITC, TxR, APC or Alexa647 in different combinations [1]. CD45 and CD3 were selected as markers for immunophenotyping. The region of interest of 5x5 fields of vision was scanned in the three color channels. The accuracy of phenotyping leukocyte subsets in the presence of DNA dye by SFM was verified by laser scanning cytometer using the same samples.
Results: All three DNA dyes yielded substantial spillover into other fluorescence channels. With increasing concentration of the DNA dyes expectedly CV-values decreased and spillover increased. DAPI showed relatively low spillover and the best CV value. These data were in agreement with those obtained on the LSC. In immunophenotyping experiments DNA served as trigger signal. DAPI and POPO-3 DNA dyes were combined with FITC and TxR or Alexa Fluor 350 and APC. These dye combinations turned out to be the best for SFM analysis. Comparable findings were obtained by FCM and LSC.
Conclusion: DNA fluorescence is applicable to identify and find leukocytes and to discriminate between

cells and platelets on the SFM. DNA content looks like a good trigger signal for leukocytes samples prepared from blood. Although DNA dyes showed strong spillover into other fluorescence channels it was still possible to immunophenotype leukocytes. DAPI showed relatively low spillover and the lowest CV value. It is therefore proposed as the best for use in the SFM system.
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P-2-138

Phase contrast signal as a trigger parameter for leukocyte measurements in imaging cytometry
Bocsi J.[1], Pierzchalski A.[1], Marecka M.[1], Malkusch W.[2], Tárnok A.[1]
[1]Heart Center, University of Leipzig, Pediatric Cardiology, Leipzig, Germany, [2]Carl Zeiss, IS GmbH, Hallbergmoos, Germany

Background: Innovative slide-based cytometry (SBC) systems lead to breakthrough in cytometry as they provide sophisticated tools for analyses of cells in tissues, culture and suspension [1]. Zeiss Imaging Solution GmbH introduced a new automated fluorescence microscope that combines imaging with cytometric features (acquiring high number of quantitative data from individual cells in multiple fluorescence channels). Moreover this technique allows for multiple cytometric analysis of the same cell after relocation. Setting the appropriate triggering signal to detect all objects on a slide is a critical step for SBC analysis. Without correct definition of the target cells the subsequent automated fluorescence collection and analysis is difficult and the meaning of obtained data is unclear. DNA staining is one of the most common triggering signals. However, the majority of DNA dyes yield massive spillover to other fluorescence channels, thus limiting their application [2]. By phase contrast signal (PCS) objects of 5-10 µm in diameter or larger can be easily distinguished by microscopy without any staining due to an optical enhancement of phase transitions between cell organelles. Aim of the study was to establish the threshold level for PCS as the trigger signal for fluorescence particles and leukocyte identification.
Methods: Axio ImagerZ1 fully motorized microscope (SFM [3]) AxioCam MRm and AxioVision software (suitable for automatic multi-channel scanning and fluorescent analysis) were used. Samples were scanned in three channels (PCS, FITC, Cy5). Beads and mononuclear cells were used. Cells were stained by antibodies labeled with FITC APC or Alexa647 in different combinations. Leukocytes stained with anti-CD45 antibodies were counted and the result was compared to the amount of PCS detected events. The proportion of leukocyte subpopulations measured with PCS as trigger signal was compared with LSC and FCM data.
Results: Focused phase contrast signals showing ring form were not optimal for the cell definition. PCS slightly out of focus allows for effective qualitative and quantitative cell analyses. Cell count triggered by PCS and CD45 was highly correlated (p=0.0003, R=0.809). PCS showed to be an accurate triggering signal for bead and leukocyte detection thus enabling cell counting and discrimination of leukocytes from platelets. Leukocyte subpopulation measurements triggered on PCS were comparable with leukocyte subpopulation distribution performed with LSC and FCM.
Conclusion: Digitized PCS is applicable for defining and finding the leukocytes and discriminate between the cells and platelets. PCS seems to be a suitable trigger signal which does not interfere with fluorescence detection in other channels.
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P-3: Cancer Biology of Solid Tumors

P-3-139

Cytokeratin 18 in determining cytotoxicity of FOLFOX and FOLFIRI chemotherapy regimes in colon carcinoma cells

Tanriverdi-Akhisaroglu S.[1], Altun Z.[1], Batu J.[2], Ates H.[3], Giray H.[4], Kocturk S.[2]
[1]Dokuz Eylul University, Institute of Oncology, Izmir, Turkey, [2]Dokuz Eylul University, Medical Faculty Department of Biochemistry, Izmir, Turkey, [3]Dokuz Eylul University, Medical Faculty Department of Hematology, Izmir, Turkey, [4]Dokuz Eylul University, Medical Faculty Department of Public Health, Izmir, Turkey

Background: The chemotherapy regimes FOLFOX and FOLFIRI used in colorectal cancer therapy are far away from satisfactory results for patients and medical oncologists. Cytokeratin-18 (CK18) is a candidate marker for determining cell death in its early period. Studies for CK18 as marker in monitoring epithelial cancers are increasing.
Aim: The aim of this study was to evaluate the relation between CK18 and apoptotic/total cell death in HCT-116 human colon carcinoma cells treated with the in vivo used FOLFOX and FOLFIRI chemotherapy regimes.
Methods: Human colon cancer HCT-116 cells were treated with FOLFOX and FOLFIRI in regard to in vivo doses. After 24h, 48h and 72 h incubation periods cell viability was determined using MTT and apoptotic/total cell death ratios were measured by Annexin-V (flow cytometry). Total soluble (M65) and cleaved epitope (M30) of CK18 were measured (ELISA) after the same time settings.
Results: EC50 doses for FOLFOX and FOLFIRI, with the doses in accordance with in vivo, were measured at 72h. Apoptosis rates measured by Annexin-V were for cells treated with FOLFOX; 24h-1.33%, 48h-2.65% and 72h-0.63% and for cells treated with FOLFIRI; 24h-5.93%, 48h-9.2 % and 72h-0.3 %. Apoptotic/total cell death ratios measured by CK18 showed correlations with the results of Annexin-V measurements.
Discussion: The cell death model that have been used in our experiments was a reliable model of apoptotic and cell growth inhibitory experiments in which in vitro applications of drug combinations showed correlations with in vivo treatment. CK18 and Annexin-V measurements and their correlations show that FOLFOX and FOLFIRI combinations cause more non-apoptotic cell death than apoptotic cell death in the colon cancer cells.
We conclude that CK18 measurement may be useful for assessing treatment effects.

P-3-140

Effects of thymidine analogues EdU and BrdU on cell viability and cell cycle progression

Diermeier-Daucher S.[1], Clarke S.[2], Bradford J.[2], Brockhoff G.[1]
[1]Institute of Pathology, University of Regensburg, Regensburg, Germany, [2]Molecular Probes-Invitrogen, Oregon, United States

Background: A recently introduced method for determining S-phase synthesis (SPF) using the nucleoside analog EdU (5'ethynyl-2'-deoxyuridine) coupled with click chemistry has been proposed as a BrdU replacement. Long term exposure of cells to BrdU has been shown to alter cell cycle progress and distribution of cell cycle phases. Structurally similar to the natural nucleoside, both EdU and BrdU modification occurs in the same region of the pyrimidine ring. Although the alkyne is unreactive in biological systems, the effect on cell viability, DNA synthesis, and cell cycle checkpoints to long term exposure has not been explored so far.
Methods: We examined the effect of long term (96 h) continuous exposure of EdU or BrdU/dC on SK-BR-3 and BT474 breast cancer cell lines. Cell cycle analysis with dead cell gating was measured on a BD LSRII (633 nm, 405 nm) using CellCycle 633-red and LIVE/DEAD® Fixable Violet cell stain. Apoptosis was

evaluated with an Annexin-V FITC/ PI Assay. Incorporation of EdU into DNA was measured with Click-iT™ EdU Alexa Fluor® 488 Cell Proliferation Assay Kit. A BD FACSCalibur (488 nm; 635 nm) was used to analyze apoptosis and EdU-incorporation.
Results: EdU treatment turned out to be applicable for cell proliferation assessment applying Click-iT chemistry. However, cell viability of SK-BR-3 breast cancer cells was highly affected by long term exposure to EdU. Media supplementation of dC with EdU did not improve cell viability. In contrast, cell viability of BT474 was not affected by EdU treatment. EdU treatment induced cell death in SK-BR-3 was due to greatly increased necrosis. Although in respect to cell viability both cell lines showed a different sensitivity to EdU: they incorporate EdU with almost the same efficiency even at low concentrations. EdU induced G2/M phase cell cycle arrest can be observed in both SK-BR-3 and BT474 cells.
Conclusion: Albeit thymidine/EdU replacement is highly appropriate for flow cytometric proliferation analysis the potential impact on cell viability needs to be evaluated. Analysis of cell proliferation using click chemistry can accurately be done using 1/200 of the standard BrdU concentration. Hence its anti-proliferative and/or necrosis inducing impact might be efficiently reduced by short time instead of continuous EdU labeling. Cytotoxicity of EdU on SK-BR-3 cells can not be attributed to increased EdU accumulation of EdU. Also BT474 cells efficiently incorporate EdU however they show significantly less necrosis. Further studies will elucidate the molecular mechanisms of EdU and BrdU induced cell death and inhibited cell cycle progression.

P-10: Clinical Cytometry

P-10-101

Is CD43 useful in the diagnosis of B-LPDS?

Apostolakis K.[1], Psarra K.[1], Kapsimali V.[1], Papasteriades C.[1]
[1]*Evangelismos Hospital, Department of Immunology and Histocompatibility, Athens, Greece*

Introduction: CD43 is a membrane glycoprotein (sialophorin), normally expressed by most leukocytes, except resting B cells. Over the last years, CD43 has been reported to provide useful information on the diagnosis of B-cell lymphoproliferative disorders (B-LPDs).
Aim: The aim of this study was to evaluate the diagnostic significance of CD43 expression in B-LPDs, by examining its expression patterns in B cells of B-LPD patients and moreover in B cells of healthy individuals and by comparing them to the expression patterns of other antigens routinely used for the diagnosis of mature B cell neoplasms.
Material and methods: A three-colour, flow cytometric analysis of a total of 104 specimens of peripheral blood, out of which 17 by healthy individuals and 87 by B-LPDs patients was performed. In particular, 63 of the patients had typical B Chronic lymphocytic leukemia (CLL), 1 Hairy cell leukemia (HCL) and the remaining 23 were identified as having other types of LPD and were grouped together under the class of Non-Hodgkin lymphomas (NHL).
Results: The study showed that in CLL B cells do usually express CD43, in contrast to NHL, where the expression of CD43 is mainly negative, as it is also in HCL. Normal B cells do not express CD43. However there were 2 cases of CLL where this antigen was not expressed by B cells and 4 cases of NHL, where CD43 was expressed. Compared to the other antigens, CD43 expression seemed to parallel to a great extent the expression of CD5. In regard to these two molecules, the immunophenotypic profile of B cells seemed to be the following: CD43-CD5- in normal B cells, CD43+CD5+ in CLL and few cases of NHL, CD43-CD5- in most cases of NHL and in HCL. There was not observed any case of CD43+CD5-, but there were observed few cases among NHL and CLL with the phenotype of CD43-CD5+. A thorough examination of the last cases of NHL revealed the diagnosis of Mantle cell lymphoma.
Conclusions: This study endorses, that CD43 does not contribute to a more precise diagnosis of CLL, since it bears the same kind of information as CD5, but it may be useful for the identification of distinct disease entities of NHL, especially in the case of CD43-CD5+ immunophenotype, which implies the presence of mantle cell lymphoma.

P-10-103

Conjugates formation and transfer of plasma membrane labelling in CD4+ primary T cells from donors and ALPS patients

Canonico B.[1], Luchetti F.[1], Arcangeletti M.[1], Biagiarelli L.[1], Ramenghi U.[2], Crescenzio N.[2], Palma F.[3], Degli Esposti M.[4], Papa S.[1]
[1]*Università degli Studi di Urbino, Dipartimento di Scienze dell'Uomo, dell'Ambiente e della Natura, Urbino, Italy,* [2]*Università di Torino, Dipartimento di Scienze Pediatriche, Torino, Italy,* [3]*Università degli Studi di Urbino, Urbino, Italy,* [4]*University of Manchester, Faculty of Life Sciences, Manchester, United Kingdom*

Fas ligand (Fast, CD95L) is a type II transmembrane protein of the tumor necrosis factor family that induces cells to send an apoptotic signal to cells expressing Fas (CD95, APO-1) (1). Defects in Fas are a cause of autoimmune lymphoproliferative syndrome (ALPS). This Autoimmune lymphoproliferative syndrome is caused by genetic mutations that interfere with apoptosis or programmed cell death, altering immmune homeostasis and resulting in an expansion of normally rare circulating lymphocytes. Although cells are usually considered as entities with relatively stable phenotypes, some physiological processes are now known that may lead to expression of unexpected cell surface. These events, known as trogocytosis, are represented by an active membrane transfer. The hallmarks of trogocytosis are the requirement of cell-to-

cell contact and possibly immune synapse formation (2). In this work we have evaluated trogocytosis in primary activated CD4+ T cells from healthy donors and ALPS patients, untreated and treated with FasL within 1h. Flow Cytometry was performed by means of PKH26, PKH67, CtxB and CFSE labelling in samples untreated and treated with two cytoskeleton inhibitors (latrunculin B and citochalasin) to ensure the phenomenon of membrane exchange. Our findings show that FasL is able to induce transfer of membrane labelling in CD4+ T cells with significant differences in healthy donors and ALPS patients. Furthermore, although in ALPS patients we didn't observe a significant phenomenon of membrane exchange, we highlight an high exposure of poshatydilserine (PS) in agreement with recent reports suggesting that PS flip-flop is linked to T-cell activation and is not an exclusive marker of early apoptosis. In conclusion our results suggest that Fas-FasL interaction induces concomitant PS exposure in donors and in ALPS patients whereas we detect a significant membrane transfer only in CD4+ cells from healthy donors.

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P-10-104

Characterization of stem cell compartment in the liver of patients with hepatocellular carcinoma

Cattaneo A.[1], Colombo F.[1], Foglieni B.[2], D'Ambrosio A.[1], Martin-Padura I.[3], Bertolini F.[3], Agliano A.[3], Rossi G.[4], Prati D.[2], Porretti L.[1]
[1]IRCCS Fond. Ospedale Maggiore Policlinico, Regenerative Medicine, Milan, Italy, [2]Ospedale A. Manzoni, Transfusion Medicine, Lecco, Italy, [3]Istituto Europeo di Oncologia, Milano, Italy, [4]IRCCS Fond. Ospedale Maggiore Policlinico, Liver Transplant Unit, Milan, Italy

Background: Recent discoveries have documented that tumors may originate from the transformation of normal stem cells, through modification of signalling pathways that regulate self-renewal in these cells. The so called 'stem cell model' of carcinogenesis suggests that cancers originate and are maintained by a rare fraction of cells called cancer stem cells (CSCs), with indefinite potential for self-renewal that drives tumorigenesis.
Aim: In this study we characterized the stem/progenitor cell compartment in intra-tumoral (IT) and extra-tumoral (ET) tissue of patients affected by hepatocellular carcinoma (HCC).
Material and methods: We analyzed 16 liver specimens from patients undergoing surgical resection. Cell suspensions were obtained with a perfusion/enzymatic digestion technique and for each patient both IT and ET hepatic tissue were evaluated. The immunophenotypic profile was defined by six-colors flow cytometry using monoclonal antibodies specific for stem and precursor markers such as CD133 (AC133/2) , which expression is described in many solid tumor types, CD34, CD90(Thy-1), CD117(c-kit) and ABCG2. We also analyzed other specific lineage markers, in particular CD45 (hematopoietic), CD146 (endothelial) and CD29 together with CD49f (hepatic). Since we were investigating very rare events, at least 1-1.5x10^6 cells were acquired. Analyses were performed only on viable cells, negative for 7 amino-actinomycin-D staining.
Results: The percentage of stem cells (CD133, CD34, CD90, CD117) was significantly higher in IT than in ET tissue (5.15±4.1% vs. 2.14±1.71%, $p<0.001$, Wilcoxon signed rank test). Particularly we found an increase of CD34 and CD90 fractions (1.08±1.57% vs. 0.41±0.38% and 2.09±2.13% vs. 0.63±0.81% respectively, $p<0.005$). ABCG2 expression was also higher in IT than in ET (0.56±0.54% vs. 0.21±0.24%, $p<0.05$). Unexpectedly, CD133 expression was not statistically different in IT than in ET specimens (1.42±1.65% vs. 0.91±1.2%, $p=0.2$). Most of CD133 positive cells co-expressed either CD146 (8.3±10.2% IT, 19.4±23.3% ET) or CD45 (61.9±32.3% IT, 61.7±28.9% ET). Moreover, about 10% of CD133 positive cells co-expressed both CD29 and CD49f antigens, without any difference between IT ad ET (10.7±8.5% vs. 9.2±3.7%, $p=0.81$).
Conclusions: Overall, patients with liver tumor have a significant expansion of IT stem cell compartment as compared to ET tissues. However, not all HCC specimens show the same antigenic profile. Cancer tissue contains significant proportion of stem cells and precursors committed to non-epithelial (endothelial and hematopoietic) lineages. Reasonably, these cells contribute to generate and maintain "accessory" tumor cells. Therefore CD133 alone cannot be used to identify genuine tumor initiating cell. ABCG2 antigen, a

stem cell marker functionally involved in determining multidrug resistance, is significantly over-expressed in IT.

P-10-105

Fetomaternal hemorrhage detection assay on flow cytometer and hematology analyzer: Automated replacement for the Kleihauer-Bettke assay

Davis B.H.[1], Davis K.T.[1], Wright D.[2]
[1]*Trillium Diagnostics, LLC, Bangor, Maine, United States,* [2]*Abbott Diagnostics, Santa Clara, CA, United States*

Despite the availability of more precise and reproducible FDA cleared flow cytometric methods for the measurement of fetomaternal hemorrhage (FMH) for nearly a decade, diagnostic practice is still dominated by use of the subjective, imprecise Kleihauer Bettke (KB) or acid elution microscopic counting assay. Interestingly flow cytometric methods have been widely adopted in the UK, Australia, and some parts of Europe. The difference in practice patterns maybe traceable to differing clinical dosing of Rh Immune globulin outside North America causing the more ready acceptance of the more accurate flow cytometric methods. Alternatively it may due to the perception that FMH assays must be available on a STAT basis and thereby representing a barrier to adoption of flow cytometric methods.
We have developed a flow cytometric method based upon anti-hemoglobin F monoclonal antibodies for use on both flow cytometers and the Abbott Cell Dyn Sapphire hematology analyzer, which has immunophenotypic capabilities. The reagent also contains propidium iodide, so as to identify nucleated cells and improve the specificity of this rare event assay. We compared the performance of this Trillium FMH detection assay for the Abbott Sapphire to the two FDA cleared flow cytometric methods (Quanti-D, Alba Biosciences, RTP, NC and Caltag (Invitrogen), Carlsbad, CA). A comparison on 72 samples ranging between 0 - 1.75% fetal cells yielded a correlation coefficient (r2) of >0.98 with no significant inter-assay bias noted. Limiting dilution studies indicated a sensitivity of < 0.05% detection of fetal red cells. The level of imprecision was comparable to the currently FDA cleared methods with a CV of <5%. The assay is a no wash technique requiring ~30 minutes to complete with less than 10 minutes of technologist time, thereby being more efficient to use than the KB assay.
The Trillium QuikQuant FMH detection assay can be performed on a STAT, 24 hour, 7 day basis and may allow for accelerated adaptation of the flow cytometric FMH technique.

P-10-106

The use of Trypan Blue as live/dead stain in flow cytometry

Eckhardt A.[1], Schmalz G.[1], Schweikl H.[1]
[1]*University of Regensburg, Department of Operative Dentistry and Periodontology, Regensburg, Germany*

Trypan Blue (TB) has been used to distinguish living from dead cells in microscopy for 50 years now. Here we describe a protocol for the adaption of TB staining in flow cytometry. TB can be measured in the APC-Cy7 channel without interfering with the FITC and PE channels. This is advantageous compared to PI staining if multiple stains are utilised.
Here, we describe a protocol for TB staining in flow cytometry using RAW264.7 mouse macrophages were treated with triethylene glycol trimethacrylate (TEGDMA), an acrylic monomer used in polymer chemistry which is known to induce apoptosis. After exposure and TB staining, cell death was indicated by a strongly increased shift in the fluorescence intensity, a second peak which is clearly distinguished from the peak of the living cells. In addition to the staining with TB, the same cells were analysed with a protocol for surface markers like CD54 and CD80, or the cells were fixed and treated further for intra-cellular staining, e.g. MAPK p38. The use of TB staining offers some practical advantages. Contrary to PI staining it is not necessary to add TB immediately prior to measuring the cells. This allows for the save preparation of the

samples in a hood. Moreover, TB staining can be combined with FITC- and PE-labelled antibodies which reduce handling time. Our method is a fast, reliable and easy-to-use technique to distinguish between living and dead cells in multi-colour flow cytometric analysis.

Supported by the Deutsche Forschungsgemeinschaft (DFG, Schw 431/11-1)

P-10-107

Quantify and quality changes of actin cytoskeleton in CHO AA8 cell line after apoptosis and mitotic catastrophe induction

Grzanka D.[1], Grzanka A.[2], Izdebska M.[2], Gackowska L.[3], Helmin-Basa A.[3], Marszalek A.[4]
[1]Collegium Medicum, Nicolaus Copernicus University, Department of Clinical Pathomorphology, Bydgoszcz, Poland, [2]Collegium Medicum, Nicolaus Copernicus University, Department of Histology and Embryology, Bydgoszcz, Poland, [3]Collegium Medicum, Nicolaus Copernicus University, Department of Immunology, Bydgoszcz, Poland, [4]Collegium Medicum, Nicolaus Copernicus University,Department of Clinical Pathomorphology, Bydgoszcz and, University of Medical Sciences, Department of Clinical Pathomorphology, Poznan, Poland

In this study we examined reorganization of the actin cytoskeleton in CHO AA8 cell line after induction different type of cell death - apoptosis and mitotic catastrophe. In this experimental model we used different doxorubicin concentrations (0.5 μM; 1 μM; 2.5 μM) for 24h.
Material and methods: Chinese hamster ovary cells (CHO AA8) were cultured in minimum essential medium eagle (MEM). The effects of doxorubicin treatment on morphology and actin cytoskeleton organization was analyzed using fluorescent and transmision electron microscopy. Flow cytometry was used for identification of cells death (TUNEL and Annexin V-FITC/7-AAD assays) and also to asses quantify changes of F-actin after cell death induction in cells and isolated nuclei (Alexa Fluor 488 conjugated with phalloidin/7-AAD).
Results: Exposition to doxorubicin caused cells death and changes in cell morphology. Anexin V-FITC/7-AAD flow cytometry analysis revealed dose dependent increase in the percentage of cells with phosphotidylserine externalisation. Cell cycle analysis revealed increase in cell polyploidy and significant increase in the percentage of cells with DNA fragmentation estimated by TUNEL assay. We observed two populations of the cells. First population consisted of rounding, shrinked and detached from the substratum also with bubbles on their surface The second population was composed of flattened and giant mono- or multinucleated cells. F-actin was demonstrated as extensive arrays network and stress fibers in giant and flattened cells. There were also seen cells with strong labeling in which the enlarged nuclei showed increased F-actin labeling comparing to other cells. In the rounded, shrinking cells strong F-actin labeling was observed in the centre of the cells as well as in the buds. On the ultrastructural level there were observed: apoptotic cells with margination and condensation of chromatin in nucleus as well as cells multinucleated or with multisegmented nuclei with distinct chromatin pattern. Moreover giant cells with intracellular small and large vacuoles and containing also electron-dense material were seen. Flow cytometry analysis reveled significant increase of F-actin expression in cells and isolated nuclei which was parallel to fluorescent microscopy observations.
Conclusions: Doxorubicin induce two different mode of cell death in CHO AA8 cell line: apoptosis and mitotic catastrophe. Cells undergoing apoptosis expressed characteristic features of that process. Cells undergoing mitotic catastrophe expressed some exclusively characteristic features and also various overlapping features of apoptosis and necrosis. We could also suggest that specific reorganization of F-actin system with its significant changes in quantity might be involved in both presented processes.
Acknowledgement: The study was supported by grant from Ministry of Science and Higher Education No 401224534

P-10-108

Complex immunophenotyping of peripheral leukocytes in chronic-inflammatory rheumatic diseases by multichromatic flow cytometry

Grützkau A.[1], Steinbrich-Zöllner M.[1], Grün J.[2], Kaiser T.[3], Raba K.[3], Wu P.[4], Rudwaleit M.[4], Sieper J.[4], Burmester G.-R.[5], Thiel A.[3], Radbruch A.[6]
[1]Deutsches Rheuma-Forschungszentrum (DRFZ), Immunemonitoring, Berlin, Germany, [2]Deutsches Rheuma-Forschungszentrum (DRFZ), Bioinformatics, Berlin, Germany, [3]Deutsches Rheuma-Forschungszentrum (DRFZ), FCCF, Berlin, Germany, [4]Charité, HU-Berlin, CBF, Dept. of Rheumatology, Berlin, Germany, [5]Charité, HU-Berlin, CCM, Dept. of Rheumatology, Berlin, Germany, [6]Deutsches Rheuma-Forschungszentrum (DRFZ), Berlin, Germany

Gene expression studies of peripheral blood cells in inflammatory diseases revealed a large array of new antigens as potential biomarkers useful for diagnosis, prognosis and therapy stratification. Generally, their validation on the protein level remains mainly restricted to a more hypothesis-driven manner. State-of-the-art multicolour flow cytometry makes it attractive to validate candidate genes at the protein and single cell level combined with a detailed immunophenotyping of blood cell subsets. We developed multicolour staining panels including up to 50 different monoclonal antibodies that allowed the assessment of several hundreds of phenotypical parameters in a few milliliters of peripheral blood. Up to ten different surface antigens were measured simultaneously by the combination of seven different fluorescence colours. In a pilot study blood samples of ankylosing spondylitis (AS) patients were compared to normal donors (ND). A special focus was set on the establishment of suitable bioinformatic strategy for storing and analyzing hundreds of phenotypical parameters obtained from a single blood sample. We could establish a set of multicolour stainings that allowed monitoring of all major leukocyte populations and their corresponding subtypes in peripheral blood. In addition, antigens involved in complement and antibody binding, cell migration and activation were acquired. The feasibility of our cytometric profiling approach was demonstrated by a successful classification of AS samples with a reduced subset of 80 statistically significant parameters, which are partially involved in antigen presentation and cell migration. Furthermore, these parameters allowed an error-free prediction of independent AS and ND samples originally not included for parameter selection. This study demonstrates a new level of multiparametric analysis in the posttranscriptomic era. The integration of an appropriate bioinformatic solution as presented here by the combination of a custom-made Access database along with cluster- and prediction-analysis tools predestine our approach to promote the human cytome project.

P-10-109

Determination of blood leukocyte concentration with constant volume acquisition volume on a flow cytometer is comparable to individualized single platform testing with beads as internal reference standard

Hansen S.[1], Dahl R.[1], Hoffmann H.J.[1]
[1]Aarhus University Hospital, Department of Respiratory Diseases, Aarhus, Denmark

Flow cytometers have a constant flow rate. This enables flow cytometers to measure leukocyte concentrations in a determined volume by acquiring data at a fixed rate over a fixed time and is called Constant Volume Acquisition (CVA). The volume aspirated by a FACS Calibur flow cytometer in 4 min at high rate was median 163 µl (IQR 156-170) with TruCount tubes. Leukocyte concentrations of 26 healthy volunteers were measured twice on up to four occasions with a Bürker-Türk chamber, by single platform technology (SPT) with TruCount tubes and on the same data set using CVA. Total leukocyte concentrations determined by CVA correlated better with measurements in a Bürker-Türk (BT) chamber than with SPT. Concentrations determined with CVA were 1, 86 % higher than with BT whereas SPT data were 5,35 % higher than BT ($p<0,001$), and 3,36 % higher than CVA ($p<0,001$). At leukocyte concentrations < 6 million/ml SPT correlated better with BT than CVA. The SPT measurement may be more variable because it

depends on measurement of the number of beads aliquoted, the number of beads and leukocytes aspirated, where both BT counting and CVA measurements only depend on the number of leukocytes counted. CVA with PanLeukoGating can be established using microscopy as a reference, and is comparable to BT chamber and SPT determination. Leukocyte concentrations can be measured with CVA on flow cytometers in research and clinical settings.

P-10-110

Artifact in multicolor labeling mimicking aberrant phenotypes

Iobagiu C.[1], Vedrine C.[1], Lambert C.[1]
[1]*University Hospital of St Etienne, Immunology Laboratory, Saint Etienne, France*

Simultaneous use of numerous new fluorochromes exposes to artifacts, especially when total blood is analyzed. The 6 color flowcytometry immuno-monitoring of total blood, occasionally revealed unexpected T cells phenotypes (CD4+ CD9+, CD4+CD56+, CD8+CD19+). The aim of this study was to characterize different types of aberrant labeling due to rare samples.
Methods: Routine analysis was performed on fresh blood using CD3-FITC, CD16-PE/CD56-PE, CD45-PerCP, CD4-PE-Cy7, CD19-APC, and CD8-APC-Cy7 on a FACScanto™ (BD Biosciences). Complementary labeling was carried out with CD8β-PE-Cy5, CD8α-APC, CD8α Alexa 647, CD25 PE and corresponding isotypes controls. The conjugates were finally tested on Cytocomp Beads.
Results: Unexpected phenotypes were found with aberrant expression of: 1 - CD4 PE-Cy7 on all CD8 APC-Cy7+ T cells; 2 - CD8 APC-Cy7 on CD4 PE-Cy7+ T cells. 3 - CD8β PE-Cy5 on CD4 PE-Cy7+ T cells. 4 - CD56-PE on CD4+PE-Cy7+ T cells. These patterns were reproduced several times of the follow-up. They were only observed by labeling of total blood but not pre-washed cells. They could be reproduced by using fractionated incriminated serum transferred on fresh cells of individuals having no artifact, or using isotypes controls captured on anti-Ig kappa coated beads. These results suggest a sensitization to fluorochromes in patients with immune disorders, and rarely in healthy individuals. The effect could be reproduced by immunizing mice with fluorochrome conjugates. The cause of sensitization to fluorochromes is difficult to understand as these molecules are not naturally encountered.

P-10-111

Immunophenotypic profile of Splenic Marginal Zone Lymphoma

Kakkas I.[1], Karmiris T.[2], Psarra K.[1], Kapsimali V.[1], Grigoriou I.[1], Delibasi S.[2], Gigantes S.[2], Pagoni M.[2], Tasidou A.[3], Rontogianni D.[4], Bakiri M.[2], Harhalakis N.[2], Nikiforakis E.[2], Papasteriades C.[1]
[1]*Evangelismos Hospital, Immunology and Histocompatibility Department, Athens, Greece,* [2]*Evangelismos Hospital, Hematology and Lymphoma Department, Athens, Greece,* [3]*Evangelismos Hospital, Pathology Department, Athens, Greece,* [4]*Evangelismos Hospital, Hematopathology Department, Athens, Greece*

Introduction - aim: Splenic Marginal Zone Lymphoma (SMZL) is a distinctive and well-characterized B-cell neoplasm that involves mainly spleen and bone marrow, recognized as an entity by the World Health Organization (WHO) classification. Peripheral blood involvement has been reported at different percentages. We present our findings from peripheral blood B-cell immunophenotype analysis of patients with SMZL with absolute lymphocytosis.
Patients and methods: We report 24 cases of SMZL with absolute lymphocytosis at diagnosis, diagnosed by histopathologic examination of surgically removed spleens. Immunophenotype analysis of peripheral blood B-lymphocytes was performed by Flow Cytometry. We used monoclonal antibodies against the following membrane antigens: CD45, CD19, CD20, CD22, CD23, CD5, CD10, CD11c, CD25, CD103, FMC-7. Surface immunoglobulins (sIg) were investigated by using FITC goat anti-κ and anti-λ polyclonal antibodies. A marker was considered as positive when expressed in more than 20% of cells above the control.

Results: The results of Flow Cytometric (FC) analysis of peripheral blood B-lymphocytes from our 24 SMZL patients were the following: CD20(+):24/24(100%), CD22(+):24/24(100%), FMC-7(+):22/24(92%), sIg(+):21/24(87%), CD5(+):4/24(17%), CD23(+):5/24(21%), CD25(+):2/24(8%), CD11c(+):5/24(21%), CD103(+):2/24(8%), CD10(+):0/24(0%). It is noteworthy that spleen and bone marrow immunohistochemical analysis of our 24 cases did not detect positivity against CD5, CD23 and CD103 antigens on clonal B-lymphocytes.
Conclusions: Our findings of peripheral blood B-lymphocyte immunophenotype analysis from SMZL patients are generally in agreement with those from previous studies. Positivity against CD20, CD22, FMC-7 and sIg was a constant finding. Positivity against CD5, CD23 and CD103 was only occasionally found. So, we believe that FC analysis of peripheral blood B-lymphocytes offers additional information concerning SMZL diagnosis.

P-10-112

NK cells receptors immunophenotype in couples with unexplained recurrent spontaneous abortions
Konsta E.[1], Psarra K.[2], Kapsimali V.[2], Kitsiou V.[2], Galaziou G.[2], Katsavria A.[2], Papasteriades C.[2]
[1]National and Kapodistrian University of Athens, Department of Chemistry, Athens, Greece, [2]Evangelismos Hospital, Department of Immunology and Histocompatibility, Athens, Greece

NK cells express a large array of surface receptors (KIRs, ILT and receptors of C-type lectin family) involved in the regulation of different NK cell functions by sensing the expression of HLA class I on potential target cells. The population of NK cells is intensely studied in the pathophysiology of unexplained recurrent spontaneous abortions (URSA).
The aim of this study was the investigation more particularly of the putative role in the pathogenesis of URSA of NK cells receptors combination in each individual couple. Materials and
Method: Peripheral blood lymphocytes from 24 couples with URSA were labeled with monoclonal antibodies CD3, CD56, CD16, CD158a, CD158b, NKp44, NKp30, NKp46, KIRp70, KARp50.3 by direct whole blood staining and analyzed by four-colour flow cytometry. The immunophenotypic study of NK surface receptors was performed on total NK cells (CD3-CD56/16+) as well as on their subsets (CD3-CD56+, CD3-CD16+).
Results: Women with URSA in comparison to men showed: a) increased NKp46 receptor positive cells as percentages of total NK cells (CD3-CD56/16+, p=0,040) as well as of their subsets (CD3-CD56+ p=0.026, CD3-CD16+ p=0.030), b) decreased incidence of CD158a and CD158b receptors regarding total NK cells and CD3-CD16+ NK cells subset. This finding was confirmed by paired t-test between women and their respective husbands data. Both, in men and women with URSA, CD3-CD56+ NK cells showed increased incidence expression of NKp46 receptor and decreased incidence expression of CD158a, CD158b, KIRp70 and KARp50.3 receptors (statistically significant) in comparison to CD3-CD16+ NK cells. This finding was not observed in a population of 25 healthy men.
Conclusions: Women with URSA in comparison to men showed increased incidence expression of natural cytotoxicity receptor NKp46 and decreased incidence of inhibitory CD158a and CD158b receptors. These disturbances in couples with URSA concern mainly CD3-CD56+ NK cells subset, a finding in agreement with other investigators implicating this subset in URSA. Further study of the interaction between NK cells receptors and respective HLA antigens in each couple could possibly clarify the role of these receptors in URSA.

P-10-113

Flow cytometry as a tool to study apoptosis of alveolar lymphocytes (AL) in interstitial lung diseases (ILD) - potential clinical importance

Kopinski P.[1], Slusarczyk-Balicka B.[2], Chorostowska-Wynimko J.[3], Pinis G.[4], Krawczyk M.[1], Szpechcinski A.[3], Szczeklik J.[2]
[1]Collegium Medicum, Nicolaus Copernicus University, Chair of Gene Therapy, Bydgoszcz, Poland, [2]Collegium Medicum, Jagiellonian University, Chair of Toxicology and Environmental Diseases, Krakow, Poland, [3]Institute of Tuberculosis and Lung Diseases, Laboratorium of Molecular Diagnostics, Warszawa, Poland, [4]Atopia NZOZ, Out-Patient Clinic, Krakow, Poland

Background: AL are crucial cells in induction and regulation of immune response to pathogens invading human lower airways. In ILD they are active in diease onset, progress and outcome. However, mechanisms responsible for AL apoptosis, including its appearance, extension and possible alterations are not clearly understood.
Methods: AL were harvested from sarcoidosis, silicosis, asbestosis, hypersensitivity pneumonitis (HP), idiopathic pulmonary fibrosis (IPF), NSIP (non-specific idiopathic pneumonitis) patients (n=89,16,19,14,35,7 resp.) and from control subjects (n=25). PS group was subdivided to Loefgren´s Syndrome (LS), chronic disease (CS) and progressive disease (PS); the subgroups of systemic steroid treated patients were identified for sarcoidosis and IPF. Apoptosis was examined by TUNEL assay, cell cycle (including sub-G1 late apoptosis peak) analysis and Annexin V staining. In order to know more about apoptosis mechanisms, TNF-R1, TNF-R2, Fas, Fas Ligand (FasL) as well as Bcl-2 family expression were evaluated. BAL supernatants were tested for TGFβ, TNFα, IFNγ, HGF, IL4, IGF1, soluble Fas (sFas) and soluble FasL levels (ELISA). BAL short term cell cuktures with subsequent cell cycle analysis were preformed in sarcoidosis and IPF materials.
Results: In nonsmoking controls average 1% of AL enters apoptosis (and 1% proliferates), according to cell cycle analysis. AL apoptosis rate was reduced in active sarcoidosis (LS, PS), HP, asbestosis (0.7 ± 0.2%; 0.5 ± 0.2% and 0.6 ± 0.3%, $p<0.05$ for all) and increased in IPF (2.3 ± 1.0%, $p<0.02$). Steroid treatment significantly enhance AL apoptosis rate and decrease AL bcl-2 expression. The subgroup of PS and IPF patients seemes to be steroid-resistant if consider AL apoptosis results. On the other hand, increased massive apoptosis was occasionally found in patients with overlapping lower airways infection and with sarcoidosis remission. Fas and TNF-α supernatant levels were negatively correlated with AL apoptosis rate (rs -0.48, $p<0.05$ and rs -31, $p<0.005$ resp.). Addition of IGF-I, but not TNF-α, to cultured BAL cells reduced the progress of AL apoptosis. Other tested factors seem have no impact on AL apoptosis; the discordant results concerning TNF system are to be explained by low TNFR1 and common TNFR2 superficial expression on AL.
Conclusions: 1. Flow cytometry used for AL apoptosis examination is useful in assessment of ILD process activity and treatment efficacy.
2. The effect of associated factors, as disease stage, tobacco consumption, concurrent infection and steroid therapy modifies AL apoptosis rate.
3. The AL apoptosis results obtained by flow cytometry support information important for assessment of ILD pathomechanisms, process activity and prognosis.

P-10-114

Commensal oral bacteria antigens prime human dendritic cells to induce Th1, Th2 OR Treg differentiation

Kopitar A.N.[1], Ihan A.[1]
[1]Faculti of Medicine, Institute of Microbiology and Immunology, Dep. of Immunology, Ljubljana, Slovenia

Periapical granulomas are usually accompanied with different anaerobic and obligatory anaerobic bacteria, which are part of normal oral flora. It is known that chronic periapical inflammatory responses occur as a

consequence of bacterial infection of this region. Dendritic cells (DC) are the main orchestration of specific responses. Immature DCs are scattered throughout the body in nonlimphoid organs and are specialized in antigen capture and processing. A number of microbial and inflammatory products activate DC wich increase expression of MHC-II and costimulatori molecules and migrate to the draining lymph nodes where they trigger T-cell stimulation. The aim of our study was to test the capacity of distinct oral bacterial antigens (prepared from Streptococcus mitis, Propionibacterium acnes and Bactroides fragilis) to prime human dendritic cells for stimulation of T lymphocyte response. For achieve T lymphocyte response the expression of CD25, CD69, intracellular IFN-g and IL-4 were determined. We prepared dendritic cells from leukocyte buffy coat from healthy blood donors. Monocytes were stimulated with IL-4 and GM-CSF and dendritic cells activated with bacterial lysates. Lymphocyte subsets that have developed in lymphocyte cultures after one week of stimulation were analyzed by flow cytometry. DCs, primed with antigens of B. fragilis have shown significantly higher activation of T lymphocytes compared to negative control. Expression of intercellular IFN-g was also significantly higher than in negative control. On the other hand the activation of lymphocytes T by P. acnes was smaller and the differentiation into T helper cells was not found. DCs presented antigens of S. mitis induced differentiation from T helper cells into mixed phenotype Th1 and Th2 subsets.
We have demonstrated, using flowcytometry, that DCs, primed with antigens B. fragilis significantly activated T lymphocytes and differentiated T helper cells in to Th1 subsets. Stimulated of lymphocytes T with S. mitis results in differentiation of T helper cells in to mixed cytokine phenotype Th1 and Th2. On the other hand DCs primed with antigens P. acnes didn´t differentiated T helper cells in to Th1 or Th2 subsets but activation of T lymphocytes with highe expression of IL-2 receptors that may have regulatory effect on infection. This findings, taken together, suggest that gram negative bacteria B. fragilis witch differenced T helper cells in to Th1-type cytokine production may caused more extended inflammation and tissue damage in periapical lesions than gram positive bacteria´s S. mitis and P. acnes witch differentiated CD4 cells in to mix phenotype or didn´t achieve differentiation in to Th1 or Th2 cytokine production. Exact knowledge of different mechanisms of T helper cells activation, which directed in the differentiation in the subpopulation of CD4 cells, will give as a better clarity of some immunopathologic processes in cronical bacterial inflammations.

P-10-115

Immunological abnormalities in CVID and XLA patients - phenotypic characteristics of lymphocytes membrane markers

Kubiszewska I.[1], Gackowska L.[1], Helmin-Basa A.[1], Eljaszewicz A.[1], Urbanska M.[1], Bernatowska E.[2], Pac M.[3], Prokurat A.[4], Michalkiewicz J.[2]
[1]Collegium Medicum, Nicolaus Copernicus University, Department of Immunology, Bydgoszcz, Poland, [2]The Children's Memorial Health Institute, Department of Microbiology and Clinical Immunology, Warsaw, Poland, [3]The Children's Memorial Health Institute, Department of Gastroentherology, Hepathology and Immunology, Warsaw, Poland, [4]Collegium Medicum, Nicolaus Copernicus University, Department of Children's Surgery, Bydgoszcz, Poland

The major principle of our research was examination of changes in immunity parameters of cellular type in patients with diagnosed immunodeficiency syndrome associated with abnormalities in humoral response. In this aim we focused on two study group fulfilling exchanged criterions: patients with diagnosed X-linked agammaglobulinemia (XLA) and common variable immunodeficiency (CVID) with diverse defect in expression of immunoglobulins. The following immunological parameters were executed:
Percentage and intensity of membrane markers in:
• populations of lymphocytes T (CD3,CD4,CD8) and NK cells (CD56).
• isoforms: CD45RA,CD45RO,CD45RB populations CD4 or CD8.
• coexpression of CD27 and CD28 receptors in lymphocytes T populations.
11 children were included in this study . The immunodeficiency was diagnosed on basis of characteristic clinical symptoms and cytogenetic studies and divided on two groups: XLA and CVID children. Eleven healthy children were served as a control. PBMCs were isolated from heparinized blood by Lymphoprep

density centrifugation. Expression of lymphocytes membrane's markers were analyzed by three-colours flow cytometry.
• Renovation process of lymphocytes T proceed: by the central renewal (dependent on thymus) and peripheral renewal (independent on thymus, called peripheral expansion).
• The characteristic feature of lymphocytes T which originate in peripheral renewal is loss of percentage of cells or density of receptors. Decreased percentage of some populations of cells (CD27+ in populations CD4+ and CD8+ of lymphocytes T), as well as individual receptors density (CD4,CD8,CD56) in CVID and, in smaller degree, in XLA groups indicates domination of peripheral expansion over central renewal of lymphocytes T.
• Considerable decrease in percentage of cells may result from high apoptosis of cells which, as literature data showed, is characteristic for cells derived from peripheral renewal.

P-10-117

Implementation and quality control of a large quantity of flow cytometry instruments for CD4 absolute and CD4% counting within HIV monitoring and AIDS patient follow-up under the national treatment program in India

Nasdala I.[1], Ost V.[1], Göhde R.[2]
[1]Partec GmbH, Partec Science & Application, Münster, Germany, [2]Partec GmbH, Partec Essential Healthcare, Görlitz, Germany

The demand for easy-to-use, accurate and affordable cell counting devices performing precise determinations of CD4+ T cell concentration for adult patients and CD4+ T cell percentages among lymphocytes (CD4%) for pediatric patients within HIV monitoring and AIDS patient follow-up is rapidly increasing. Many countries especially in Africa, Asia and Latin America have started to significantly scale up the national treatment programs for HIV/AIDS patients.
CyFlow Counter and CyFlow SL instruments have been designed for securing efficient and cost-saving operation even under very difficult environmental and infrastructural conditions and are therefore well suited also for use in rural areas and resource-poor settings far away from central laboratories. Since 2002, already 800 CyFlow CD4 counting devices have been successfully implemented in Africa and Asia, covering more than 500,000 patients at cost of approximately USD 2 per test, while using previously introduced techniques in such countries showed average test cost of USD 40 (WHO, 2002).
India has an estimated population of 1.1 billion and 2,470,000 people living with HIV - the prevalence rate is 0.36% (UNGASS Country Progress Report 2008). The National AIDS Control Organization (NACO) under the government of India and in cooperation with the United Nations Office for Project Services (UNOPS) has recently procured 67 CyFlow Counter instruments for supporting the nationwide HIV monitoring program.
A key issue for laboratory instrumentation is the internal and external quality control and quality assessment which should be performed regularly and documented well. The cost (labour and material) should be kept at a possible minimum. It is shown that QC/QA can be performed highly economical for the CyFlow technique using precounted control material with approved concentration (Count Check Beads green, Partec, Germany) and blood samples prepared for CD4 and CD4% counting using dedicated IVD approved and CE marked reagent kits with monoclonal antibodies (CD4 easy count kit and CD4% easy count kit, Partec, Germany).
For the first time ever in flow cytometry, a QC/QA program including an evaluation of the intermachine variation was run in parallel with such a large quantity of instruments. Within only 4 days, all 67 CyFlow flow cytometers have been installed in India and checked for their general performance and counting accuracy using first the same approved lot of precounted control bead material and subsequently three different blood samples stained in bulk for CD4 analysis. The coeffient of variation (CV) was 4.7% for CD4 absolute count results and 3.3% for the control bead material on all 67 instruments. This result evidences a high degree of reproducibility and standardization in flow cytometry as well as instrument manufacturing which is especially important for considerations in context to the targeted large scale increase of national treatment programs in the global fight against HIV/AIDS.

P-10-118

Evaluation of circulating plasma cell count kinetics in the treatment of relapsed/refractory myeloma

Peceliunas V.[1], Matuzeviciene R.K.[1], Janeliuniene M.[1], Griskevicius L.[1], Kucinskiene Z.A.[1]
[1]*Vilnius University Medical Faculty, Vilnius, Lithuania*

Flow cytometry has applications of diagnosis, predicting outcome and evaluating of treatment efficacy in malignant hematological disorders including Multiple Myeloma(MM). There are many well established prognostic factors other than flow cytometry in predicting outcome of MM. One of newer emerging prognostic factor in MM might be the number of circulating plasma cells (CPC) in peripheral blood.
Objectives:
1. To identify and calculate circulating peripheral blood plasma cell population(s) in relapsed/refractory MM patients.
2. To evalua te the kinetics of peripheral blood CPC count in response to treatment as an early predictor of outcome in MM patients.
Methods: Flow cytometric analysis of peripheral blood and bone marrow of 22 adult MM patients. Inclusion criteria: refractory/relapsed myeloma patients, salvage treatment with bortezomib based regimen or VAD, signed informed consent. Samples prepared by whole blood lysis technique using six-color staining: CD45/CD19/CD38/CD138/CD56/CD20/c-kappa and c-lambda. CPC count established before and 3-4 weeks after first cycle of chemotherapy (before second cycle). Samples were analyzed with FACSCanto (BD) 6 colour flow cytometer. For cell acquisition a “live gate” was set on CD138+/CD38+ cells population and up to one million events were acquired. Data was analyzed by sequential gating method using FACSDiva software.
Results: In all MM patients CPC cells consisted of two populations: aberrant CPC (CD38(+), CD138(+), CD 19(-), CD56(+), CD45(-/+) Kappa or Lambda clone and normal CPCs (CD38(+), CD138(+), CD 19(+), CD56(-), CD45(+). Kappa(+)Lambda(+)). Phenotype of plasma cells subsets (aberrant and normal) were the same in PB and BM in all patients.
We were able to detect CPCs (normal or/and aberrant immunophenotype) in 86% (19 /22) patients before treatment and 95% (21/22) after treatment. We found that aCPC reduced significantly after one treatment course (p=0.011). Mean aCPCs before and after treatment respectively 9,8×10e-4 and 3,6×10e-4 There was a trend to increase in counts of immunophenotypically normal CPC after one treatment course (p=0.146). Mean nCPCs before and after treatment respectively 9.6×10-4 and 1,9×10e-3.
Total CPC (aberrant + normal immunophenotype CPCs) did not differ before and after one treatment course. Median nCPCs before and after treatment respectively 1.9×10e-3 and 2.2×10e-3.
According to aCPCs dynamics during treatment we defined three groups of patients: no aberrant CPCs detected, aCPCs reduced and aCPCs not reduced.
Conclusions:
I. CPC’s carrying normal and aberrant immunophenotype respond to chemotherapy differently.
II. Total CPC count did not differ before and after one treatment course.
III. It is necessary to establish both normal and aberrant immunophenotype CPC populations using CPC count as a marker of response to treatment.
IV. Further studies are needed to establish prognostic significance of CPC kinetics in response to myeloma treatment

P-10-119

Study of variability of absolute T lymphocytes subsets counts in HIV peripheral blood by adoption of a single platform

Rebordao M.M.[1], Wilkening C.[2], Lago J.L.[1], Carvalho E.[1], Silva M.[1]
[1]*Hospital Militar de Belem, Laboratorio de Analises Clinicas, Lisboa, Portugal,* [2]*Harvard School of Public Health, Centre for Biostatistics in AIDS Research, Boston, United States*

Measuring CD4 and CD8 counts in HIV-positive patients remains the single most important immunological parameter measured in HIV-infected population for evaluation of their prognosis, immune deficiency status, response to therapy and diagnosis of AIDS.
CD4 and CD8 can be determined on a flow cytometer by either single or dual platform technology.
Recent recommendations propose that single-platform technology should be the gold standard for CD4 measurement because it offers better inter-laboratory coefficients of variation and a significant improvement in the assay precision.
The aim of this study is to evaluate the variability between dual platform and the single-platform for CD4 and CD8 count immunophenotyping .
Dual-platform technology uses a haematology analyser (Abbott Cell Dyn 3700) to obtain a total white cell count and lymphocyte absolute count. CD4 and CD8 absolute values are then calculate from CD4 and CD8 percentage positive results obtained from flow cytometer (Coulter Epics XL). Single platform technology uses latex-beads of a predefined concentration, which are added to the blood sample immediately before flow cytometric analysis without need to use an additional analyser. We studied CD4 and CD8 from 90 different HIV-positive patients. The CD4 counts were divided in 3 groups: CD4<200, 500<CD4>200, CD4>500.The results variability, when compare the two methodologies, are shown in the table 1:

T lymphocyte subsets	Number samples	Median dual method value	Percentiles of single minus dual platform differences (counts x 10 6/L)		
			10th%	50th% (Median)	90th%
CD4<200	30	146	-23	0	21
CD4 200-500	22	364	-39	16	53
CD4>500	38	710	-129	-17	105
CD8	90	864	-148	5	138

The statistical estimation showed the numbers of cells we can take more or less in the different 10th, 50th and 90th percentiles due solely to the technique change. Any change bigger than this probably involves some biologic change.
We conclude that change in methodology can introduce some variability in enumeration of absolute T lymphocytes subsets but with this report the clinics will be able to understand what are the biologic changes or technique change with or without modification of clinical approach.

P-10-120

Five color minimal residual disease detection in acute myeloid leukemia based on initial diagnosis
Rinota E.[1], Psarra K.[2], Kapsimali V.[2], Grigoriou I.[2], Papasteriades C.[2]
[1]National and Kapodistrian University of Athens, Department of Chemistry, Athens, Greece, [2]Evangelismos Hospital, Laboratory of Immunology and Histocompatibility, Athens, Greece

Introduction: Diagnosis and monitoring of acute myeloid leukemia (AML) is based on flow cytometry immunophenotype in addition to other methods. Among more sensitive methods, multiparameter flow cytometry is applied to quantify the degree of minimal residual disease (MRD).
Monitoring of MRD is based on the detection of aberrant phenotypes of leukemic cells. Applying multi-colour flow cytometry, results in a higher specificity of the LAIP (leukemia associated immunophenotype) determination as well as higher sensitivity for discrimination of leukemic from normal cells.
Aim: The aim of this project was MRD determination in AML cases using diagnostic immunophenotype and five colour flow cytometry.
Methods and materials: 25 AML patients were studied. Taking into account four colour immunophenotype of diagnosis (CD45, CD3, CD4, CD8, CD19, CD10, CD20, CD34, CD38, CD22, CD7, CD2, CD56, CD36,

CD64, CD14, CD15, CD16, CD11b, CD117, CD33, CD13, HLADR, MPO, LF) as well as the type of leukemia according to FAB classification, a combination of five fluorochrome conjugated monoclonal antibodies (FITC, PE, ECD, PC5, PC7) is created. Antigens used in each combination were characteristic of patient's individual leukemic phenotype.
Results: Out of 25 patients, 22 (88%) displayed asynchronous antigen expression, 2 (8%) cross-lineage antigen expression and 1 (4%) antigen overexpression. Therefore, after complete four colour immunophenotype, in each follow-up sample a unique five colour combination was applied. Antibodies that were used were characteristic of abnormal cell population at diagnosis. No change of the initial immunophenotype was noted. In addition the combination HLADR-CD117-CD34-CD33-CD45 was applied in all samples for normal blast cells determination arising from bone marrow regeneration after chemotherapy. In 38% of patients MRD was not detected, MRD level of $1*10^{-4}$-$1*10^{-3}$ was noted in 20%, $1*10^{-3}$-$1*10^{-2}$ in 27% and more than $1*10^{-2}$ in 15%. In two cases, one month after detection of MRD >10^{-3}, patients relapsed.
Conclusions: The application of a five antibodies unique combination, taking into account the diagnostic immunophenotype, gives the possibility for more sensitive discrimination beetween residual leukemic cells and normal ones. MRD quantification at this level provides valuable information for therapeutical strategies.

P-10-121

Data-driven compensation for 2 color flow cytometry
van Rodijnen N.[1], Hoop S.[1], Nap M.[1]
[1]*Atrium Medical Centre Heerlen, Pathology, Heerlen, Netherlands*

Aims: Simultaneous application of different fluorchromes in flow cytometry introduces spectral cross-over (S), which causes an intensity dependent bias in the acquired counts. Compensation for cross-over is hard to standardize because it is typically based on visual interpretation of logarithmically displayed data, which makes it subjective and error-prone. All current compensation algorithms need manual intervention. We present an algorithm for post acquisition compensation which we call Data Driven Compensation (DDC) and does not need any manual intervention.
Methods: The essence of the DDC algorithm is that it automatically selects counts in flow cytometry list mode files with zero primary fluorescence (ZPF). In 2 color flow cytometry these counts are defined as the counts of which the combination of the acquired fluorescence for both fluorchromes are the same for a negative control (NC) and the corresponding test sample (TEST). These counts are called the common counts (CC) and are used by the DDC algorithm to calculate the S value. A NC contains in majority ZPF counts. The counts in the TEST are the same as those of the NC, supplemented with positive counts. The CC of a NC and TEST will not include these positive counts, for the simple reason that they appear solely in the TEST.
Since the CC contain in majority ZPF counts, their variance is caused by three factors only: auto fluorescence, noise, and cross-over. Auto fluorescence and noise are independent of the signal of a secondary fluorchrome, while S causes the total fluorescence to increase proportional to the intensity of this secondary fluorchrome. This difference between "signal independence" en "signal dependence" makes that the slope of the line through the median of the CC is a good measure for the amount of cross-over. Using this measure, compensation may be done automatically. For example; in 2-color flow cytometry with single cross-over, a count is present in both the NC and TEST with an acquired value for the primary fluorchrome of 40 and a value of 800 for the secondary fluorchrome, the compensation value for this CC is then 40/800=0.05.
Results: We compared the cross-over values generated with the DDC algorithm with manual determined compensation values of 159, two color acquisitions with single cross-over. The found regression line (Smanual= 0.90*SDDC+0.0049) revealed a good match between the manual compensation values and the ones generated by DDC. The small scatter around the regression line (RMSE = 0.0034) indicates that DDC generates compensation values that are comparable with those determined manually.
Conclusions: We conclude that the DDC algorithm allows for fully automatic compensation and may

support the standardization of compensation for spectral cross-over. Future work will address application to 2 color flow cytometry with double cross-over and the use for the CC as reference points, for automatic selection of positive counts in a test sample.

P-10-122

Detection of complications following solid organ transplantation by CD64 on PMNs

Kamprad M.[1], Grey D.[1], Bauer K.[1], Knaack H.[1], Fangmann J.[2], Emmrich F.[2], Sack U.[1]
[1]University of Leipzig, Clinical Immunology and Transfusion Medicine, Leipzig, Germany, [2]University of Leipzig, Departement of Visceral, Transplant, Thoracic-and Vascular Surgery, Leipzig, Germany

Background: Polymorphnuclear cells (PMN) play a key role in inflammatory reaction preventing severe infections. Namely following solid organ transplantation, immunosuppressed adoptive immune mechanisms make cells of the innate defense system to key players. Activity of PMN can be monitored by quantifying the expression of CD64 (CD64/PMN). The purpose of this study was to evaluate the diagnostic efficiency of CD64/PMN for identification of ingoing complications in the postoperative course of solid organ transplanted patients.
Material and methods: We included 25 kidney transplanted, 13 liver transplanted and 4 pancreas-kidney transplanted patients. For each patient, a immunophenotyping was performed including expression of CD64 by PMN, NKp44 by NK cells, and HLA-DR by monocytes, respectively, furthermore CD4/CD8-ratio, leucocyte count, and CRP. CXCR3 has been quantified on NK-cells, CD4+T cells, and NKT-cells. Starting with praeoperative values, we evaluated the course of these markers for 3 months and calculated sensitivity, specifity, and Youden index.
Results: Clinical signs for any complications such as infections, rejections, surgical complications, cardiac complications and post-ischemic transplant-pankreatitis were observed at 29 measurement points. By using ROC curve analysis we could confirm the existing cut-off value of > 1.5 for CD64/PMN for detecting serious complications in postoperative course of transplanted patients. All patients with a combined kidney-pancreas tranplant presented values higher than 1.5 which seemed to be caused by a high operative and inflammatory trauma. The Youden index representing diagnostic efficiency for detection of complications was shown to be .58 for CD64/PMN and hereby superior to monocytic HLA-DR expression (.38) or CXCR3 on NKT (.37).
Conclusion: We conclude that detection of CD64 on PMNs is a powerful tool to detect complications in transplated patients at a very early timepoint.

P-10-123

The importance of clonality studies as a tool for the differential diagnostic between de novo and secondary acute myeloid leukemia

Santos-Silva M.C.[1], Fernandez C.G.[2], Rasillo A.[2], López A.[2], Montero J.F.[2], Jara-Acevedo M.[2], Sanchez M.[2], Orfao A.[2]
[1]Universidade Federal de Santa Catarina, Análises Clínicas, Florianópolis, Brazil, [2]Universidad de Salamanca, Centro de Investigación del Cáncer, Salamanca, Spain

Background and objective: Acute myeloid leukemia (AML) includes a heterogeneous group of disorders characterized by clonal expansion of myeloid blasts in bone marrow (BM), peripheral blood (PB) and/or other tissue. The progressive clinical course and outcomes for patients with secondary AML have been poor compared to people who develop de novo AML. Meanwhile, the distinction of de novo AML from secondary AML still remains a challenge particularly when a prior subclinical MDS is suspected. The goal of this study is to evaluate the utility of the analysis of clonality for distinguishing myeloid cells from patients diagnosed with de novo AML with and without multilineage displasia.
Design and methods: A total of 27 consecutive patients (13 males and 14 females) diagnosed with either

de novo AML (n=25) or de novo biphenotypic acute leukemia (n=2) were included in the present study. Distribution of patients according to the WHO criteria was as follows: acute promyelocytic leukaemia with t(15;17) (n=4); AML with t(8;21); minimally differentiated AML (n=4); AML without maturation (n=4); AML with maturation (n=2); acute monoblastic leukaemia (n=3); acute monocytic leukaemia (n=2); acute myelomonocytic leukaemia (n=3); and plasmacytoid dendritic cell leukemia (n=1). Isolation and purification of specific BM- and PB-derived cell populations was performed using a FACSAria flow cytometer (BDB). Clonality studies were performed on purified blast and differentiated myeloid cells population using the human androgen receptor gene (HUMARA) assay in case of females (n=14) and the presence of chromosome alterations by interphase fluorescence in situ hybridization (iFISH) in male patients (n=13).
Results and conclusion: Overall 7 out of 13 females studied displayed a monoclonal HUMARA assay pattern and/or cytogenetic abnormalities as detected by interphase iFISH for all population of myeloid cells analyzed, as well as blast cells; one case showed homozygotic pattern and 2 cases showed a polyclonal pattern. 3 out of the 12 males studied by iFISH showed cytogenetic alterations in common in both the compartment of blast cells and mature/maturing myeloid cells, while in the other two cases the cytogenetic abnormalities found were restricted to the blast cell compartment. Thus, all together 10/25 cases presented clonality in different subpopulations of maturing myeloid cells, whereas this did not occur in 5 patients; in other 10 cases the results were not conclusive, such cases corresponding to males without cytogenetic alterations, or females with an homozygotic HUMARA assay and no cytogenetic markers by iFISH or with not enough cells to perform the HUMARA assay/FISH studies. Our results suggest that systematic analysis of clonality by the HUMARA assay and/or iFISH can be important for identification of clonal involvement of maturing myeloid cells in patients with secondary AML, and contribute to differentiation of de novo AML.

P-10-124

Long-term culture of rat bone marrow-derived neural stem cells leads to a gradual loss of stem cell markers expression

Sarissky M.[1], Balik V.[2], Sulla I.[2]
[1]*Faculty of Medicine/P.J. Safarik University, Central Laboratory of Clinical Cytometry, Kosice, Slovakia,*
[2]*Faculty of Medicine/P.J. Safarik University, Dept of Neurosurgery, Kosice, Slovakia*

Aims: Neural stem cells (NSCs) derived from the adult bone marrow represent a potentially promising source of neural progenitors for replacement therapy after central nervous system damage. The aim of the present study was to evaluate the effect of long-term cell cultivation on the immunophenotype of rat bone marrow-derived NSCs.
Methods: Bone marrow cells were harvested from rat femoral bones and cultured in alpha-MEM medium supplemented with 10% ES-FBS, 2 mM L-glutamine, 10 ng/ml rat LIF and 10 ng/ml human LIF. At passage 4, cultures were split in two parts. The first one continued with the same medium whereas the second one received additional supplementation with human FGF-b and EGF. The immunophenotype of the cells was analysed at different cell culture time points by flow cytometry using a combination of monoclonal antibodies targeted against the following membrane and intracellular differentiation markers: rat CD90-FITC /rat Nestin-PE / rat CD45-PE-Cy5.
Results: After initial 14 days (passage 0), a heterogeneous culture of adherent cells was obtained. Twenty-five per cent (range 21.2%-30.3%) of the cells displayed phenotype (CD45-, CD90+++, cyNestin++) compatible with that of NSCs. During subsequent cultivation, cells started to gradually lose the expression of both CD90 and cyNestin. The mean fluorescence intensities (MFIs) of 9320, 9356, 9636, 9250 and 8238 or 3620, 3614, 2681, 1628 and 1104 were observed for CD90 or cyNestin at passages P0, P7, P12, P15 and P19, respectively. This loss of stem cell markers was even more pronounced when cells were cultured in the medium containing human FGF-b and EGF: the MFIs observed were 9320, 9552, 8772, 6671 and 6916 or 3620, 3605, 1737, 541 and 485 for CD90 or cyNestin at passages P0, P7, P12, P15 and P19, respectively.
Conclusions: Cultivation of rat bone marrow cells in the appropriate culture media leads to the generation of cells that display phenotypic characteristics typical for NSCs. However, these phenotypic characteristics

are gradually lost during long-term cell culture.
Acknowledgement: This work was supported by the Slovak Research and Development Agency under the contracts No. APVT-20-014002, APVT-20-032504 and APVV-20-052005 as well as by grants VEGA 1/3361/06 and VVGS 58/2006.

P-10-125

Immunophenotypical characteristics of B-cell chronic lymphocytic leukaemia with 11 deletion by iFISH

Sarissky M.[1], Jakubovova M.[1], Bernatova S.[2], Hajikova M.[1], Kafkova A.[3], Stecova N.[3], Guman T.[3], Fricova M.[3], Tothova E.[3]
[1]Faculty of Medicine/P.J. Safarik University, Central Laboratory of Clinical Cytometry, Kosice, Slovakia, [2]Labmed, a.s., Central Laboratory of Clinical Cytometry, Kosice, Slovakia, [3]Faculty of Medicine/P.J. Safarik University, Dept of Haematology and Oncohaematology, Kosice, Slovakia

Aims: The clinical course of chronic lymphocytic leukaemia (CLL) is extremely variable with survival times ranging from 2 to over 20 years from initial diagnosis. During the last decade, a number of novel clinical and biological factors of prognostic significance have been identified in CLL including specific chromosomal abnormalities such as deletions of 13q14 and 11q22.3-q23.1, trisomy 12, deletions of 6q21-q23, and deletions/mutations of the TP53 at 17p13. Here, we analysed a possible relationship between the deletion of 11q22.3 and expresion of CD markers encoded at 11q as well as other CD markers.
Methods: Twenty-two consecutive CLL patients in various stages of the disease were included in the study. The median age of the patients was 65.7 years (range 47-81). The expression of CD5, CD10, CD11c, CD19, CD20, CD22, CD23, CD24, CD25, CD27, CD34, CD38, CD43, CD45, CD56, CD79b, CD103, CD185 (CXCR5), CDw210 (IL-10R), FMC7, mIgD, mIgM, mIg-kappa, mIg-lambda and cyBcl-2 was analysed by 4-coulour flow cytometry. In each patient, the presence of del(11q) was evaluated by interphase FISH using the LSI ATM (11q22.3) SO Probe. The analyses were performed on mononuclear cells isolated by Ficoll-Urografin density centrifugation, frozen in FBS/10%DMSO and stored at -80°C.
Results: Deletion of 11q22.3 was detected in 18.2% (n=4) of CLL patients. As compared to cases with normal 11q status, patients with del(11q) displayed a significantly lower expression of CD11c (p=0.038), CD20 (p=0.027) and CDw210 (p=0.019), i.e. antigens encoded on 16p11.2, 11q12-q13.1 and 11q23.3 chromosomal loci, respectively. No association was observed between del(11q) and the expression of the remaining CD markers.
Conclusions: Deletion of 11q22-q23 is one of the most frequent chromosomal abnormalities associated with adverse prognosis in CLL. Here, we found that del(11q) is associated with reduced expression of CD11c, CD20 and CDw210. The present data need to be confirmed in larger series of patients. Nonetheless, CDw210, CD11c and CD20 could be considered as potentially useful markers for the identification of CLL cases with 11q deletion.
Acknowledgement: This work was supported by the Slovak Research and Development Agency under the contracts No. APVT-20-032504 and APVV-20-052005, and by the League Against Cancer, Slovakia (Liga proti rakovine SR).

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PD Dr. Susann Müller
Group leader Flow Cytometry
Department of Environmental Microbiology
Helmholtz Centre for Environmental Research - UFZ
Permoserstr. 15
04318 Leipzig, Germany

Phone: +49 341 235 1318
Fax: +49 341 235 2247
susann.mueller@ufz.de
http://www.ufz.de

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Dr. M. Arroz (course) and Dr. C. Lambert (conference) as chairs

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