

Day 2: Wednesday, February 11th
Cell-Based Applications

9:00 – 12:00 Morning Symposium: Grace Auditorium

Capturing Signalling Events in the Immune System *in Situ*

Presenter: Margaret Harnett, Ph.D., University of Glasgow, Scotland, UK



*Dr. Harnett is Professor of Immune Signaling at the Division of Immunology, Infection and Inflammation at University of Glasgow's Biomedical Research Centre. Her research career has been in the field of Immune Cell Signalling, with initial interests in the mechanisms underlying regulation of neutrophil degranulation and respiratory burst dysfunction in immune deficiency. Her work led to the identification of a novel site of G-protein-regulated exocytosis (Ge) and the discovery that a defect in the respiratory burst of autosomal recessive patients with chronic granulomatous disease reflected the failure to phosphorylate a key component of the NADPH oxidase complex. She then became interested in the nascent field of lymphocyte signalling at the National Institute for Medical Research, London, where she produced the first data on early signalling events in the G-protein mediated-regulation of B cell antigen receptor (BCR) signalling, identifying the key regulatory elements involved both in coupling the BCR to lipid signalling pathways under mitogenic conditions and in their desensitisation during negative feedback inhibition by immune complexes. Returning to the University of Glasgow she established a group to study the signalling mechanisms underlying development of the immune response. Her research at the University has primarily focused on dissecting the differential signalling mechanisms associated with functional maturation of the immune response and its evasion by pathogens in order to identify novel targets for therapeutic intervention in autoimmune inflammatory disease. Over the last five years a particular focus has been to translate the analysis of intracellular signalling mechanisms from the test-tube to the *in situ* physiological environment of the immune response in animal models.*

Abstract

Understanding the molecular mechanisms and cellular interactions that regulate both the induction and the phenotype of immune responses is central to the development of safe, efficacious therapies to combat infections and inflammatory disorders. However, until recently it has not been possible to analyse physiologically relevant interactions *in situ*, as the technology has not been available to directly visualize and functionally correlate the key molecular and cellular events underpinning immunity and tolerance in the intact immune system. Recent advances in quantitative imaging technology, such as laser scanning cytometry (LSC) and new methodologies in the analysis of cell signalling, now allow analysis of such signalling and functional events *in situ*. Indeed, we have used these approaches to show that the distinct functional outcomes of priming and tolerance are associated with marked differences in the amplitude, kinetics and cellular localisation of activated, phosphoERK signals at the level of individual antigen-specific T cells. Moreover the GTPase, Rap1, which can antagonize the generation of such phosphoERK signals and has previously been reported to accumulate in tolerant cells, exhibits an inverse pattern of expression to phosphoERK in individual antigen-specific primed and tolerized T cells. Thus, while phosphoERK is expressed by more primed than tolerized T cells, Rap1 is expressed by higher percentages of tolerant compared with primed Ag-specific T cells following induction of priming and tolerance *in vivo*. Our analysis suggests that the maintenance of tolerance of individual antigen-specific T cells *in vivo* may reflect the recruitment of up-regulated Rap1 to the immune synapse, potentially resulting in sequestration of Raf-1 and uncoupling of the TcR from the RasERKMAPKinase cascade.

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Analysis of DNA Damage Response by Cytometry

Presenter: Zbigniew Darzynkiewicz, M.D., Ph.D., Brander Cancer Research Institute, New York Medical College, Valhalla, NY



Zbigniew Darzynkiewicz is Director of the Brander Cancer Research Institute at New York Medical College and Professor of Pathology, Medicine and Microbiology/Immunology at the same College. Formerly he was a Member of the Sloan-Kettering Institute for Cancer Research and Professor of Cell Biology and Genetics at Cornell University Medical School. He received his M.D. and Ph.D. degrees from the Medical School of Warsaw and completed his post-graduate studies at the State University of New York at Buffalo and at the Medical Nobel Institute of Karolinska Institute, Stockholm, Sweden.

Dr. Darzynkiewicz's research focuses on cancer cell growth and the regulatory mechanisms associated with cell proliferation, apoptosis and sensitivity to anti-tumor drugs. He developed several techniques to analyze metabolic parameters related to cell cycle kinetics, prognosis of tumor progression, DNA damage and apoptosis that have world-wide application. He is the past President of the Cell Kinetics Society and the International Society for Advancement of Cytology. He is Editor, Co-editor or a member of the Editorial Board of 20 scientific journals.

Abstract

DNA in live cells undergoes continuous damage caused by endogenously generated oxidants and by environmental genotoxins. Because repair of damaged DNA is error-prone, the defects accumulate with age, contributing to cell senescence and preconditioning to neoplasia. Effectiveness of most anticancer drugs relies on the extent of DNA damage that they induce in cancer cells. The assessment of the extent of DNA damage is thus of great importance in many disciplines of biology and medicine.

The sensitive reporters of DNA damage are activation of ATM (A-ATM) through Ser1981 phosphorylation, phosphorylation of histone H2AX (P-H2AX) on Ser139, activation of Chk2 through Thr68 phosphorylation (A-Chk2) and phosphorylation of tumor suppressor p53 on Ser15. We have used phospho-specific antibodies to these proteins combined with multiparameter cytometry to correlate their phosphorylation with activation of caspase-3 (A-C3) and cell cycle position, the events reporting induction of apoptosis and cell cycle effects, respectively. Laser scanning cytometry (LSC) was particularly useful to evaluate the extent of DNA damage that involved formation of DNA-double strand breaks.

This multiplexed approach was applied to measure DNA damage induced by several exogenous genotoxins and metabolically generated oxidants. Exposure of cells to UV-B induced P-H2AX concomitant with A-C3, maximally in early-S phase, with no activation of ATM. DNA topo1-inhibitor topotecan triggered A-ATM, P-H2AX, A-Chk2 and A-C3 only in S-phase cells, with maximum at mid-S. DNA topo2-inhibitors mitoxantrone and etoposide induced A-ATM, P-H2AX and A-Chk2 in all phases of the cycle, with maximum in G₁; A-C3, however, was seen in S-phase cells only. Replication stress caused by aphidicolin, hydroxyurea or thymidine led to P-H2AX and A-C3 (in S-phase) but did not trigger A-ATM. The genotoxins from tobacco smoke caused A-ATM, P-H2AX and A-Chk2 primarily in S-phase cells.

Constitutive A-ATM and P-H2AX were seen in untreated normal or tumor cells and they reflected the ongoing DNA damage caused by metabolically generated oxidants. The level of constitutive A-ATM and P-H2AX correlated with cells metabolic rate and was many-fold higher in mitogenically stimulated- than in G₀- lymphocytes. Extent of constitutive A-ATM and P-H2AX, which was maximal during S and G₂, was reduced by antioxidants and ROS scavengers (vitamin C, N-acetyl-cysteine, COX-2 inhibitors), metabolic inhibitors (2-deoxyglucose, 3-bromopyruvate) and growth at hypoxia. Analysis of constitutive A-ATM and P-H2AX provides the sensitive means to measure effectiveness of agents such as anti-oxidants or caloric restriction mimetics which, through neutralizing radicals or lowering aerobic metabolism, protect DNA from the damage.

The multiparameter cytometry to concurrently measure A-ATM, P-H2AX, A-Chk2, A-C3 and DNA content revealed a wealth of information on association between DNA damage, recruitment of DNA repair machinery, activation of cell cycle checkpoints and induction of apoptosis. The capability of LSC to enumerate immunofluorescent (IF) foci of P-H2AX, A-ATM and A-Chk2 as well to measure maximal pixel in addition to the integrated fluorescence over cell nuclei provided a highly sensitive instrumentation tool in analysis of constitutive DNA damage by endogenous oxidants.

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Regulation of Cell Cycle Transitions

Presenter: James Jacobberger, Ph.D., Case Western Reserve University and Case Comprehensive Cancer Center, Cleveland OH



Since 1985, James Jacobberger has been on the faculty of Case Western Reserve University where he is now Professor of General Medical Sciences (Oncology), and Associate Director for Shared Resources and Director of the Cytometry and Imaging Microscopy Core of the Case Comprehensive Cancer Center. He has specialized in cytometry as a scientific discipline from his doctoral studies at the University of Rochester onwards, currently focusing on cell signaling and cell cycle processes viewed from a systems orientation. Current research is centered in two enterprises—multi-variate cell cycle analysis and modeling, and leukemia cell signaling—with a long-range goal of creating an analytical system, preferably within a clinical pathology or basic cell biology setting, in which the measurements made at the cytometer are interactively interrogated by the investigator and a computer program with mathematical models of cell biochemistry and integrated systems simulations running underneath.

Abstract

We understand analysis of cytometric DNA content from first principles. Univariate data derive from and resolve to a kinetic expression profile of DNA content over time. Cells exist in G1 at one genome; synthesize a second genome during S, and exist at two genomes for G2+M. This profile is a three-segment compound linear equation (flat – rising – flat). This same dynamic, ordered chemical process is true for any cell cycle regulator or regulated biochemical activity, although the oscillations may be more complex. The correlative power of multiparametric cytometry, coupled with the asynchronous nature of cells¹, provides a platform to generate complete and precise expression profiles for an unlimited number of biochemical activities. Because analysis sets “expression” for each parameter within a set of ordered time “bins” that are equal for all parameters, all parameters are correlated whether or not they came from the same sample. Therefore, theoretically, these expression profiles can be correlated across samples and across measurement platforms. This enables cytometry assays of true N-dimensionality. For example, flow cytometry provides unlimited sampling depth (numbers of cells within a reasonable period of measurement time) and higher parameter number; laser scanning cytometry provides molecular localization and compartment size. This ability to cross platforms and create correlated, localized concentration expression profiles for an unlimited number of parameters provides a platform of unprecedented power for support and testing of mathematical models of cell biochemical networks based on systems of ordinary differential equations (ODE).

¹ Which means that cells exist at all discrete states within the possible multi-parametric data space; therefore, the kinetic expression profile will have representative measures at each “step” in time.

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**Cell-based Assays to Address Toxicology Issues in Pre-Clinical
Drug Development Using Laser Scanning Cytometry**

Presenter: Padma Narayanan, D.V.M., Ph.D., Amgen, Inc.



Dr. Narayanan's research career has focused on understanding the role of reactive oxygen and nitrogen intermediates in cellular pathophysiology. As a doctoral student at Purdue under Paul Robinson, he used cytometric approaches to gain insights into neutrophil and endothelial patho-physiology on exposure to endotoxins and environmental toxins. Later at the Los Alamos National Laboratories, he broadened the scope of this investigation to understand the role of oxidative stress in radiation-induced DNA damage, silicosis and chronic beryllium disease. Subsequently, in several positions within the pharmaceutical industry, he has integrated cytometric technologies and cellular patho-physiological endpoints into identification and characterization of drug-induced pharmacologic/toxicologic responses of potential drug candidates at different stages of development.

Abstract

Laser scanning cytometry (LSC) is widely used for immunofluorescent techniques on monolayers of cultured cells and tissue sections. In addition, LSC enables re-interrogation of single cells for further investigation via brightfield and fluorescence microscopy.

In this presentation, we demonstrate LSC use to support a mitochondrial toxicity-based lead optimization study. Mitochondrial dysfunction has been increasingly implicated as a mechanism for drug-induced toxicity. Screening for mitochondrial dysfunction prior to lead optimization is being conducted by several pharmaceutical companies in lieu of cytotoxicity assays. We selected 40 compounds known to induce various organ toxicities related to mitochondrial dysfunction *in vivo*. Compounds were categorized into four major groups: mitochondrial respiratory chain inhibitors, oxidative stress inducers, mitochondrial polymerase inhibitors, and various kinase inhibitors. In addition, a number of compounds that did not induce mitochondrial toxicity were chosen as negative controls. An LSC-based platform using human hepatocellular carcinoma cell line HepG2 cells was set up to simultaneously monitor mitochondrial membrane potential (MMP) change (using potentiometric dye TMRM) and cell viability (using calcein-AM). Each parameter was calculated for individual compounds. Our result demonstrated that the LSC-based assay increased the probability of predicting mitochondrial toxicity in a cell-based screening system.

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**How Laser Scanning Cytometry Opened a Door to Study
Cellular Inflammation and Apoptosis in Chronic Lung Disease**

Presenter: Andrew J. Halayko, PhD, Manitoba Institute of Child Health & Department of Physiology, University of Manitoba, Winnipeg, Manitoba



Andrew Halayko is Associate Professor in the Departments of Internal Medicine, Physiology, and Pediatrics & Child Health at the University of Manitoba and Director of the Asthma/COPD Research Centre in the Section of Respiratory Diseases, Department of Medicine. Dr. Halayko's research has been instrumental in building current concepts of phenotype plasticity of airway smooth muscle and its potential role in airway remodeling and asthma pathogenesis. He has recently implemented a translational research approach using transgenic mouse models to investigate mechanisms orchestrating airway inflammation, remodeling, and hyperresponsiveness. His current research interests focus on the role of caveolins as determinants of lung mesenchymal cell responses, and the potential for statins and other inhibitors associated with the mevalonate cascade to treat obstructive airway disease. Dr. Halayko is the author of nearly 90 peer-reviewed articles.

Abstract

Asthma is associated with chronic airways inflammation that underpins acute “asthma attacks” and fixed airway obstruction through progressive structural remodeling of the airways. Airway remodeling includes thickening of airway smooth muscle (ASM), due to myocyte hyperplasia and hypertrophy, encircling the bronchi. As direct evidence for excessive proliferation for ASM is sparse, inhibition of physiological apoptosis may contribute to ASM thickening. Persistent inflammation of the airways can stem, in part, from suppressed apoptosis and increased proliferation of infiltrating inflammatory cells, such as eosinophils, which characterize asthma-associated inflammation. Due to small sample sizes availability, quantitative analyses of the inflammatory cell population in the airways of humans or murine disease models is limited to subjective light microscopy-based histological cell counting of bronchial lavage cytospins. A real limitation is the lack of ability to quantify inflammatory cell differentials *in situ* or using lung lavage specimens and for quantitative assessment of proliferation and apoptosis in a cell-specific basis. To meet these needs we have developed two approaches using an iCyte[®] Laser Scanning Cytometer (LSC). First, with antibodies for murine major basic protein (MBP), an eosinophil marker, we established protocols to assess eosinophil number in cytospin samples from lung lavage of mice challenged with allergen to induce asthma-like inflammation. We subsequently applied this protocol to measure eosinophil number *in situ*, using murine lung specimens. In a second study, using transformed and primary cell lines we established LSC protocols to discriminate apoptotic cells using DNA fluorescence (Hoechst 33342; integrated and maximum pixel) in combination with cleaved-caspase 3 detection. We compared this approach with FACS-based methods using PI-uptake and determined the latter is rather less sensitive in detecting early apoptosis *in vitro*. We confirmed the utility of our LSC approach *in situ* by comparing the apoptosis index in ASM (identified by sm- α -actin staining) and eosinophils (identified by MBP labeling). Collectively our experiments establish protocols that enable *in situ* assessment of apoptosis and proliferation in a cell specific manner, making these techniques valuable tools for future assessment of key biological process in precious samples from human asthmatic subjects.