

# ELISPOT technology: The latest tricks

The BioPark Hertfordshire, Welwyn Garden City, AL7 3AX: 15<sup>th</sup> October, 2009

09:00 – 09:30 **Registration**

09:30 – 9:45 Introduction by the Chair: *Prof. Paul Lehmann*, Case Western Reserve University Cleveland, USA

9:45 – 10:15 **Dual-color ELISPOT: theory and praxis**

*Dr Alexey Karulin*, Cofounder, Vice President of R&D, Cellular Technology Ltd., Cleveland, OH.  
Dual color (DC) ELISPOT assays permits measuring the secretion of two cytokine by a single cell. DC ELISPOT assay is important when saving cell material is a concern or when cytokine coexpression is to be established. While DC assays using UV detection (UV-Spot) are still in the final phase of development, DC ELISPOT assays with chromogenic substrates we pioneered are mean while well established. In this talk I will cover my work comparing the sensitivity of DC ELISPOT assays with intracytoplasmic staining, concluding, that if DC ELISPOT performed and analyzed properly, the results are essentially identical. I will also elaborate on basic theoretical considerations for DC ELISPOT analysis, and cover important technical aspects, such as how to: choose substrates that provide clearly distinguishable red and blue spots, set up proper controls, and how to discriminate actual co-expression from random overlay of single color spots.

10:15 – 10:25 **Introduction to the Elispot/Fluorospot Bioreader (R) 5000**

*Wolf Zeller* CEO BIO-SYS GmbH, Germany

10:25 – 10:45 **Validation of the Elispot/Fluorospot Bioreader (R) 5000**

*Werner Freber*, CEO BIO-SYS GmbH, Germany

Comparing to other biomedical assays, the Elispot assay has not gone to Pharmaceutical routine for a long time. Based on the speakers own experience suggestions are given how to validate a Bioreader 'on site', by comparing other user groups approach. Additionally we will get into details about the history of developments and the capabilities of modern automated reader systems. Special focus is given on the problem solution to de- agglomerate clusters of spots by use of the unique, patented Bioreader illumination system.

10:45 - 11:00 **Speakers Photo**

11:00 - 11:30 **Coffee break**

11:30 – 12:00 **Simultaneous detection of two or more cytokines by FluoroSpot**

*Dr Niklas Ahlborg*, Assoc. Prof. Head of R&D, Mabtech, Sweden.

The development of FluoroSpot has enabled a more unambiguous detection of cells secreting either, or both, of two cytokines. Fluorospot can thus be used to identify cells secreting multiple cytokines, e.g. polyfunctional T cells producing IFN-gamma and IL-2 as well as to distinguish between cell populations such as Th1 & Th2 cells, Th1 & Th17 cells, etc. Since the analysis is based on separate filter systems for the respective fluorochromes used for detection, FluoroSpot also facilitates the development of assays for more than two cytokines. Functional dual FluoroSpot applications and prospective triple cytokine assays will be discussed

12:00 – 12:30 **Using ELispot to detect rare antigen specific T cells**

*Dr Sefina Arif*, Guys Hospital, London

There is an inability to access target tissues in many autoimmune diseases and hence one has to focus on peripheral blood in order to get a snapshot of what is occurring in the target tissue. Elispots are highly sensitive assays and can be used to detect low frequency T cells in peripheral blood, In addition, because of the array of cytokines that can be detected, it is possible to gain some insight into the nature of the pathological response and to monitor this response during immunomodulatory therapies.

12:30 - 13:30 **Lunch and Poster Viewing**

13:30 – 14:00 **ELISPOT Assays Provide Reproducible Results Among Different Laboratories for T-Cell Immune Monitoring – Even in Hands of ELISPOT-Inexperienced Investigators**

*Dr Wenji Zhang*, Senior Staff Scientist, Cellular Technology Ltd, Cleveland, OH

Measurements of antibodies in bodily fluids, e.g., by ELISA, have provided robust and reproducible results for decades and such assays have been validated for monitoring of B-cell immunity. In contrast, measuring T-cell immunity has proven to be a challenge due to the need to test live cells in functional assays ex vivo. Several previous efforts looking into the reproducibility of ex vivo T-cell assays between different laboratories, or even within the same laboratory, have provided rather discouraging results. The hypothesis we tested here is that those poor results are due to the lack of assay and data analysis standardization, rather than the inherent complexity of T-cell assays. In this study, eleven laboratories across Europe and the United States were provided identical reagents and were asked to follow the same protocol while testing aliquots of the same three cryopreserved peripheral blood mononuclear cells (PBMC) in an interferon- $\gamma$  (IFN $\gamma$ ) ELISPOT assay measuring the antigen-specific T-cell response to a CMV peptide. The data showed that ELISPOT assays provided highly reproducible results among different laboratories when the assay procedure and data analysis is standardized. Since ELISPOT assays have been qualified and validated for regulated studies, they are ideal candidates for robust and reproducible monitoring of T-cell activity in vivo.

14:00 – 15:00 **Troubleshooting Panel Discussion**

15:00 – 15:30 **Afternoon Tea/Coffee and viewing of exhibitor booths; last poster viewing**

15:30 – 16:00 **Quantification of IFN- $\gamma$  produced by human purified NK cells following tumor cell stimulation: comparison of three IFN- $\gamma$  assays**

[\*Eva Lion\*](#), Vaccine and Infectious Disease Institute, University of Antwerp, Belgium

Natural killer (NK) cell-derived IFN-gamma has become a subject of major interest, given its importance in bridging the innate and adaptive immune system. To date, only limited data are available on IFN-gamma detection methods using purified NK cells. In this study, we determined whether there is a correlation between NK cell-derived IFN-gamma following tumor cell stimulation measured using the enzyme-linked immunosorbent assay (ELISA), the enzyme-linked immunospot (ELISPOT) assay and intracellular cytokine staining (ICS) assay. Our results reveal that the choice of IFN-gamma detection method can markedly influence the outcome regarding induction of NK cell IFN-gamma by tumor cells.

16:00 – 16:30 **CTL' ImmunoSpot platforms for studies of ELISPOT multiplexing.**

*Drs Wenji Zhang and Alexey Karulin*, Cellular Technology Ltd, Cleveland, OH

Expression of different cytokines as well as the coexpression of cytokines can be either studied using fluorescent detection reagents, or by the dual color enzymatic approach. During my talk, I will first cover the basic principles of single color ELISPOT analysis, that is, how to count accurately the frequency of antigen-specific T cells that produce cytokine avoiding subjectivity in setting counting parameters or gates. Subsequently, I will extend these principles to dual color counting. Finally, CTL's latest ImmunoSpot platform will be presented that is capable of automated six color analysis.

The presentation will include live demonstration of, and work with the ImmunoSpot software, as well as a concluding question answer session.

16:30 – 17:00 **Chairman's summing up**

### About the chair

Prof. Paul V. Lehmann trained as a T cell immunologist. He introduced and patented image analysis for ELISPOT (United States Patent No 08/577,957) dedicating 40 of his 100 publications to the basics of ELISPOT, including single cell resolution, per cell productivity, cognate vs. bystander cytokine, T cell avidity measurements, determinant mapping etc. In 1998, he founded CTL to assist scientists in ELISPOT analysis. CTL offers GLP-compliant ELISPOT contract research, ELISPOT readers (visible light and UV), PBMC libraries and reference samples, as well as serum free test media. Prof. Lehmann is the President and CEO of CTL.

### About the Speakers

Dr. Wenji Zhang is a senior staff scientist at CTL. She has dedicated the last five years to ELISPOT standardization. Since serum is a major viable in ELISPOT assay performance, she has developed serum free PBMC freezing and thawing protocols, as well as test media. She developed human PBMC Reference Samples with defined antigen reactivity levels and cytokine signatures. Moreover, she has been a key player in defining the user requirements for the SmartCount function in CTL's ImmunoSpot software that automatically establishes scientifically validated counting parameters thereby eliminating subjectivity from ELISPOT analysis

Dr Niklas Ahlborg is Director of Research and Development at **Mabtech AB**, a Swedish biotech company focusing on the development and production of ELISpot kit and reagents. Background in academic research on parasite immunology, vaccine development and B- and T-cell immunology at Stockholm University and Edinburgh University. Assoc. Professor at Dept of Immunology at Stockholm University

Werner Freber Graduated Master chemical Engineering / Darmstadt / Germany. Development engineer at Grace / USA .Product manager of New Brunswick Sci / Fisher Sci. / Biotronic. Founder of BIO-SYS since 1986 Developer of Bioreader. The Biomedical plate analyzer.

Eva Lion obtained a Master in Biomedical Sciences in 2006 at the University of Antwerp and is currently a PhD student in the Laboratory of Experimental Hematology at the Vaccine & Infectious Disease Institute of the University of Antwerp in Belgium. Eva's research is focused on the immunobiology of human natural killer cells and their interaction with leukemic tumor cells and myeloid dendritic cells, from a cancer immunotherapeutic viewpoint. She is currently investigating the effect of Toll-like receptor-agonist-modified leukemic tumor cells on NK cell functions, on which she has recent papers and several abstracts on international conferences.

Dr Alexey Karulin - 1989 Lomonosov Moscow State University, Ph.D. in Chemistry. 2004-present Cellular Technology Ltd., Cleveland, OH. 1993-2004 Case Western Reserve University, Cleveland, OH. 1989-1993 Bach Institute of Biochemistry, Russian Acad.Sci., Moscow, Russia

Dr Sefina Arif studies T cells in type 1 diabetes with the current focus on the detection of Th17 cells in type 1 diabetes using the Elispot assay. She has extensive experience of the ELISpot assay including the use of this assay to monitor patients undergoing immunotherapy and to discriminate between patients and controls in blinded studies



## FULL ABSTRACTS AND POSTERS

### **QUANTIFICATION OF IFN- $\gamma$ PRODUCED BY HUMAN PURIFIED NK CELLS FOLLOWING TUMOR CELL STIMULATION: COMPARISON OF THREE IFN- $\gamma$ ASSAYS**

E Lion, ELJM Smits, ZN Berneman and VFI Van Tendeloo

*Vaccine and Infectious Disease Institute (VIDI), Laboratory of Experimental Hematology, Faculty of Medicine, University of Antwerp (UA), Antwerp University Hospital (UZA), Wilrijkstraat 10, B-2650 Antwerp, Belgium. [eva.lion@ua.ac.be](mailto:eva.lion@ua.ac.be)*

Interferon (IFN)- $\gamma$  released by natural killer (NK) cells has become a subject of major interest, given its importance in bridging the innate and adaptive immune system. Interestingly, reports concerning tumor cell stimulation of NK cells show divergent data on which stimuli induce IFN- $\gamma$  production. Here, the question remains whether tumor cell recognition is sufficient to trigger IFN- $\gamma$  or whether a second signal is required such as type I IFN. While IFN- $\gamma$  detection methods are abundantly used with peripheral blood mononuclear cells or purified T cell fractions as responder populations, only limited data is available about comparison of these assays with purified NK cells. In this study, we assessed the relationship between stimulation of human purified resting peripheral blood NK cells with one (tumor cell or IFN- $\alpha$ ) and two (tumor cell + IFN- $\alpha$ ) signals by measuring IFN- $\gamma$  using three different assays. We performed the enzyme-linked immunosorbent assay (ELISA), the enzyme-linked immunospot (ELISPOT) assay and intracellular cytokine staining (ICS) assay in parallel per donor and determined whether there was a correlation between these assays.

Our results show that two-signal stimulation of human resting NK cells induces significantly more IFN- $\gamma$  compared to one-signal stimulation, readily picked up by all assays. Moreover, statistical analysis points towards a positive correlation between these assays for IFN- $\gamma$  produced following two-signal stimulation. Importantly, we show that tumor cell stimulation alone is enough to trigger secretion of IFN- $\gamma$ , but this finding was only evidenced by ELISPOT. These results reveal that the choice of IFN- $\gamma$  detection method can markedly influence the outcome regarding induction of NK cell IFN- $\gamma$  by tumor cells.

### **THE EFFECT OF APOPTOTIC CELLS ON VIRUS-SPECIFIC IMMUNE RESPONSES DETECTED USING IFN-GAMMA ELISPOT**

K Lenders<sup>1</sup>, B Ogunjimi<sup>2</sup>, Ph Beutels<sup>2</sup>, P Van Damme<sup>2</sup>, ZN Berneman<sup>1</sup>, VFI Van Tendeloo<sup>1</sup>, ELJ Smits<sup>1</sup>

<sup>1</sup>*Laboratory of Experimental Hematology, Vaccine & Infectious Disease Institute, University of Antwerp, Antwerp University Hospital, Wilrijkstraat 10, B-2650 Antwerp, Belgium*

<sup>2</sup>*Centre for Health Economics Research and Modeling Infectious Diseases (CHERMID) & Centre for the Evaluation of Vaccination (CEV), Vaccine & Infectious Disease Institute, University of Antwerp, Universiteitsplein 1, B-2610 Antwerp, Belgium*

#### Introduction:

The use of cryopreserved peripheral blood mononuclear cells (PBMC) allows the determination of virus-specific immune responses in large-scale vaccination trials by the sensitive ELISPOT technique. Generally, viability of PBMC after thawing is determined using DNA-intercalating agents, e.g. 7-amino-actinomycin D (7-AAD) or propidium iodide (PI), to identify dying or dead cells that lost membrane integrity. However, cell viability can be determined even more accurately when also a marker of apoptosis is included, e.g. annexin V. In this study, we wanted to assess the influence of apoptotic cells on the magnitude of virus-specific responses against different forms of antigens of varicella-zoster virus (VZV)- and cytomegalovirus (CMV) using IFN-gamma ELISPOT.

### Materials and Methods:

Apoptosis was induced in thawed PBMC by UV irradiation. After an overnight resting phase, viability of the UV-irradiated and non-irradiated thawed PBMC was determined by flow cytometry after staining with 7-AAD and Annexin V (FITC-conjugated). A gradient of apoptotic cells was added to a variable or fixed number of non-irradiated PBMC of the same donor and overnight IFN-gamma ELISPOT was performed on these PBMC in the presence of viral antigens. The antigens used for stimulation were cell lysates of MRC-5 cells infected with VZV or CMV, as well as a CMV peptide pool.

### Results:

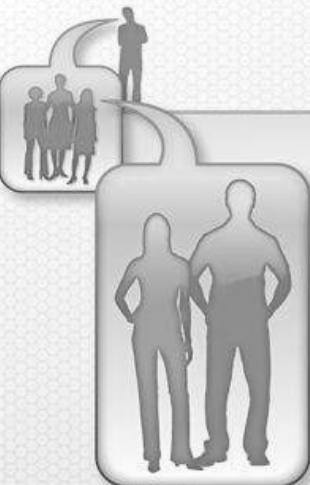
In our experiments, we observed that stimulation of UV-irradiated PBMC with viral antigens did not lead to spot-forming cells (SFC) in IFN-gamma ELISPOT. When the fraction of UV-irradiated apoptotic cells was gradually increased within a total of  $2.5 \times 10^5$  PBMC per well, the number of SFC in response to VZV- or CMV-antigen decreased, as expected because of the decrease in viable spot-forming PBMC. However, the VZV-specific response also decreased when increasing the amount of UV-irradiated apoptotic cells added to a fixed number of viable PBMC per well in the presence of VZV lysate. This decrease in the number of spots was less pronounced when PBMC were stimulated with CMV lysate and absent when using a CMV peptide pool as the source of antigen.

### Conclusion:

In conclusion, we showed that the presence of apoptotic cells in a population of thawed PBMC can affect virus-specific IFN-gamma responses detected by ELISPOT. This effect was dependent both on the type of virus and on the type of antigen used for stimulation. Therefore, we recommend using highly viable (defined as both 7-AAD- and annexin V-negative) fractions of thawed PBMC in ELISPOT for an accurate detection of immune responses in vaccination trials.

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## **NOVEL CANCER IMMUNOTHERAPY TARGETS AMONG SPAG PROTEINS – EXPRESSION PATTERN AND AUTOANTIBODY DETECTION USING PHAGE DISPLAYED ANTIGEN MICROARRAY**

*K. Silina, L. Ivanova, P. Zayakin, Z. Kalnina, I. Meistere, A. Line  
Latvian Biomedical Research and Study Centre, Ratsupites str. 1, Riga, LV-1067, Latvia;*

The identification of cancer associated and immunogenic proteins is still a challenge in order to improve antigen targeted antitumour immunotherapy approaches. The so called cancer-testis (CT) antigens have proven themselves to be one of the most perspective group of proteins to target patient's immune system against as normally they are present only in immunoprivileged tissues and are strongly immunogenic when faced if faultily expressed by tumour cells. The group of proteins called sperm associated antigens comprises 15 members (SPAG1, 2, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 15, 16, 17). They are functionally and evolutionary distinct proteins with a common expression in testis or sperm and the ability to elicit immuneresponse leading to infertility. Lately also a role in tumourigenesis has been shown for several SPAG proteins and the application of SPAG9 and SPAG10 in cancer immunotherapy has been proposed, hence SPAG proteins might represent a novel group of CT antigens with possible implications in anti-tumour immunotherapy. The aim of this study was to determine if any of the SPAG proteins might be a potential novel candidate for immunotherapy approaches by assessing their expression in various normal and cancerous tissues and their antigenicity by detecting SPAG antibodies in sera of various cancer patients.

Initially the open source expression databases were mined as well as potentially cancer associated alternative splice variants were analysed by using a bioinformatics tool developed in our laboratory to select most promising candidates for experiments. Expression analyses were performed using RT-PCR, real time RT-PCR (qPCR) and IHC. QPCR expression data were normalized using a normalization factor calculated from the expression level of three most stable reference genes for each cDNA sample. To detect antibodies in patients' sera, we cloned SPAG proteins together with other well-known CT antigens like NY-ESO-1, MAGE A1 etc. in the T7 phage expression system. 47 antigen carrying and 9 empty phage clones were purified and printed in triplicate on glass slides creating phage displayed antigen microarrays which were screened with 223 sera from melanoma, limpholeucosis, gastric, breast, lung, colorectal, thyroid and prostate cancer (each around 30) patients' and 30 healthy donors' sera. The serum IgG presence was detected by Cy5 conjugated secondary anti human IgG antibody. The amount of printed phage per spot was monitored using a mouse anti phage tail antibody and detected by a Cy3 conjugated anti mouse IgG antibody. The Cy5/Cy3 signal ratios were used to define serum response intensity.

Initial data mining and RT-PCR revealed that SPAG2, 4, 5, 7, 9, 10, 11, 12, and 13 are well expressed in various normal tissues. Bioinformatic analyses of alternative transcripts revealed a potential testis and/or cancer associated splice variants (sv) for SPAG9 and 17. QPCR among 14 various normal tissues showed a testis selective expression for SPAG6, 8, 16 and 17sv, and testis restriction for SPAG15, while SPAG1 and 9sv were abundantly present in several normal tissues other than testis. IHC was done for SPAG6, which showed its presence in 50% of lung cancer specimens and all adjacent normal lung tissues of lung cancer patients while lung tissues of healthy individuals were negative. mRNA analyses in different tumour-normal tissue pairs showed that SPAG6, 8, 16, and 17 were overexpressed in cancer specimens, while SPAG15 was present only in trace levels in a few samples. Phage displayed SPAG6, 8, 9sv, 16, and 17 were spotted on the antigen microarray and screening analyses showed the presence of antibodies against SPAG8, 16 and 17 exclusively in sera of cancer patients and no healthy donors.

Our data suggest that previously proposed immunotherapy targets SPAG9 and 10 as well as SPAG1, 2, 4, 5, 6, 7, 11, 12, and 13 are not be suitable candidates for immunotherapy approaches due to their abundant presence in various normal tissues, while SPAG8, 15, 16, and 17 might be considered for further investigation as immunotherapy targets due to their restricted expression profile and potential ability to elicit spontaneous humoral immune response in cancer patients.

This meeting was organised by Euroscicon ([www.euroscicon.com](http://www.euroscicon.com)), a team of dedicated professionals working for the continuous improvement of technical knowledge transfer to all scientists. Euroscicon believe that they can make a positive difference to the quality of science by providing cutting edge information on new technological advancements to the scientific community. This is provided via our exceptional services to individual scientists, research institutions and industry. The event was hosted by 'BioPark' ([www.biopark.co.uk](http://www.biopark.co.uk)), a research and development centre in Welwyn Garden City providing specialist facilities and support for bioscience and health technology businesses to grow, and to develop new products and technologies