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Summer school in flow cytometry for immunology: report from a successful ESCCA experience

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ABSTRACT

INTRODUCTION

Immunology is an important scientific discipline in constant development. It has evolved in one of the most important health-related biological sciences and it contributes to a lot of medical specialities. Its study is central to the development of many biological treatments and it constitutes an integral part of personalized medicine.

Cytometry in general, and flow cytometry in particular, plays a central and absolutely fundamental role in either clinical or research-oriented immunology labs. In these last decades, flow cytometry is constantly evolving and offers numerous opportunities to scientist trying to decipher the immunological status of patients or their response to treatments.

Among the goals of the European Society for Clinical Cell Analysis (ESCCA) is the dissemination of education regarding the applications of cytometry. Summer Schools in flow cytometry applications for immunological investigation may represent exceptionally effective educational tools for students and professionals working in cytometry labs in Europe and throughout the world. The educational program of the schools is focused on both cytometric and immunological issues and most importantly on their combination. The environment of the Summer Schools is also crucially important in offering, during the course, the opportunity of friendly interaction between teachers (educators) and students. Greek islands represent the optimal location for such a school. A cool shady room where knowledge is disseminated, followed by exposure to the sun, sea, and good food all together create "The School", as imagined by the ancient Greek philosophers.

The 1st ESCCA Summer School in Flow Cytometry for Immunology, organized by Katherina Psarra and Silvia Della Bella took place in the Greek island of Kos on June 19th-23rd, 2019.

EDUCATIONAL CURRICULUM

A good knowledge of all the innate and adaptive immune cell types is very important. Therefore, immunophenotyping in immunology regarding all cell types, including T and B lymphocytes, NK cells, innate lymphoid cells, dendritic and other myeloid cells, classical and myeloid derived suppressor cells, were thoroughly covered. Typical changes occurring in immunopathologic condition, as in primary immune deficiencies, were demonstrated. Functional assays aimed at assessing essential cell functions, including cell proliferation, apoptosis, cytokine production, cytotoxicity, degranulation, phagocytosis and killing, were also explained and illustrated. An overview of the educational program of the 1st ESCCA Summer School in Flow Cytometry for Immunology is summarized in Table 1, and briefly reported hereafter.

IMMUNOPHENOTYPING IN IMMUNOLOGY

T cells

Immune system is built up in order to distinguish self from non-self, to protect the organism from pathogenic or non-pathogenic elements, which are recognized as foreign and destroyed after having been sensitized (memory), and finally to keep tolerance towards specific autoantigens.

T cells are the key components of the adaptive immune system and mediate what is otherwise known as cellular immunity. Therefore the aim of this educational topic was to understand the main steps of maturation and differentiation of the protagonist, the T cell.

During development, T cell progenitors migrate from the bone marrow to the thymus, where they expand under the influence of IL-7 and begin to express the T cell receptor (TCR). At the stage of full expression of the TCR, the majority of T cells (90%) carry the αβ receptor type on their surface. A small percentage however, <10% carry the γδ receptor type. Studies of the T lymphocyte diversity in immunodeficiencies and in diseases with a pathological immune background offer a better understanding as well a diagnostic tool in immunology. Unlike αβ T cells, γδ T cells display a restricted TCR repertoire. They are located in peripheral blood (PB), intestine, skin, spleen, and lymph nodes where they act as a link between innate and adaptive immunity because they lack precise major histocompatibility complex (MHC) restriction. The γδ TCR recognize non-peptide antigens and they provide a wide range of defense mechanisms against microorganisms.

Further down, trained and differentiated T cells are positively or negatively selected to express either the CD4 or CD8 coreceptor, in addition to

Table 1 Educational program of the ESCCA Summer School in Flow Cytometry for Immunology (June 2019 – Kos Island, Greece)

Immunophenotyping in immunology	T cells
	B cells
	dendritic cells
	NK cells
	innate lymphoid cells
	suppressor cells
Functional assays by flow cytometry	cell proliferation
	cell apoptosis
	cytokine production
	phagocytosis
	oxidative burst
	autophagy
	cytotoxicity
	basophil degradation
	flow x-match
Flow cytometry in health and disease	flow cytometry in primary immunodeficiencies
	BAL immunophenotyping
	circulating tumor cells
	immunomonitoring during treatment with biological drugs

their TCR. The inability to express antigen receptors at any stage leads to apoptosis. Although, the mature T cell pool is commonly recognized on expression of either CD4 or CD8, rare populations of double positive or double negative T cells can be found.

Coming into contact with antigens triggers their differentiation into effector and memory cells. NaïveCD3+CD45RA+CD45RO-CCR7+CD62L+,central memory CD45RA-CD45RO+CCR7+CD62L+, effector memory CD45RA-CD45RO+CCR7-CD62L- and effector T cells CD45RA+CD45RO-CCR7-CD62L-.

Furthermore, distinct populations of CD4+ and CD8+ can be identified based on the type of cytokines that they secrete. During the presentation, the processes and pathways involved in the development of T cell were analysed. The specific receptor-ligand pair interactions which mediate the homing, proliferation, survival, and differentiation were explained, showing flow cytometric data and possible pitfalls during flow cytometry analysis. [1, 2, 3]

B cells

Basic B cell immunophenotyping was covered in a 2 hour lecture, in which we tried to unravel the mysteries of B cell development in the bone marrow and of antigen specific B cell maturation in the spleen and secondary lymphoid organs. Instead of listing the expression patterns of the basic B cell markers, we tried to explain the currently perceived role of each molecule in B cell physiology and to describe its fluctuations throughout the B cell immune response [4]. This way, we provided the participants the information that would give them insight to the function (or the history) of the cells that express each specific marker, rather than strictly define the B cell subset classification, given that the latter is actually a human intervention in a yet to be fully discovered world. However, as practice requires precision, we showed the basic gating strategies for the study of B cells by flow cytometry and referred to common pitfalls and discrepancies in their enumeration [5]. We also mentioned the basic concepts, techniques and some applications of the study of antigen specific B cells [6]. Finally, in order to link the B cell study to clinical practice and to underscore the importance of certain molecules in B cell development, we showed different abnormal B cell patterns in specific defects causing primary immunodeficiencies [7, 8].

Dendritic cells (DCs)

DCs are ubiquitous professional antigen-presenting cells that play a crucial role in initiating and shaping immune responses. The effects of DCs on adaptive immune responses depend partly on functional specialization of distinct DC subsets, and partly on the activation state of DCs, which is largely dictated by environmental signals. In particular, whereas fully mature activated immunostimulatory DCs promote immune responses, immature DCs or DCs matured in immunosuppressive conditions counteract T-cell activation.

After providing an overview on the biology of DCs and the functional specialization of distinct DC subsets, we illustrated the strategies more commonly used for the identification of DCs in the peripheral blood, which represents the most accessible source of human DCs. In fact, because DCs lack unique lineage markers, their identification relies on possible different combinations of positive and negative markers. The importance of characterizing the activatory/inhibitory phenotype of DCs and the pattern of their cytokine production in different clinical settings was explained. We started showing the characterization of peripheral blood DCs (pb-DCs) performed by using a 3-color approach [9], and increased progressively complexity, showing a 6-color [10] and finally an 18-color [11] approach to the study of these cells.

Advantages and disadvantages of these different approaches were discussed. Tips and tricks related to analysis of rare cells, use of polychromatic flow cytometry, and DC-specific behavior were also presented. Finally, in order to introduce multidimensional unsupervised analysis of flow cytometric data, exemplificative t-distributed stochastic neighbor embedding (tSNE) analysis showing different subsets of pbDCs were shown.

Natural killer (NK) and innate lymphoid cells (ILCs)

In the last decade, innate lymphoid cell (ILC) family has grown enormously, demonstrating to be deeply involved not only in fighting viral infection and tumors but also in lymphoid tissue formation, in tissue regeneration and showing to possibly play a key role in many immune and autoimmune diseases [12].

These ILC populations, NK cells excluded, have not yet been unequivocally characterized and many recent studies revealed that they display a high degree of plasticity thus allowing their prompt adaptation to environmental change [13]. For these reasons, we summarized in a simple and unquestionable way what, up to now, we know for sure about ILCs phenotype and functions but most of all we tried to explain how to approach these cells from a rational and critical perspective, fundamental to avoid taking all these “brand new” discoveries for granted [14].

Moreover, we tried to give the students a deep overview of NK cells history starting from their discovery in the late seventies and ending up now in this exiting era of immune therapy based oncological treatments.

As a matter of fact, nowadays, NK cells relevance in human health preservation is showing more and more entirely new and fundamental facets [15,16]

Suppressor cells

Human body has innate immune regulatory mechanisms, but in addition it has evolved an external system of regulatory mechanisms in order to be protected from autoimmunity and to avoid in general immune harm. These cells called suppressor cells are the classical ones, including many subpopulations with different receptors, different mechanisms of action acting at different stages of the immune response.

These classical suppressor cells include:

a) NKT cells, a small population recognizing glycolipids presented by CD1d through polymorphic TCRs and secreting IL-4 and IL-10,

b) Tregs, (natural and inducible Tregs) CD4+CD25 highFOXP3+CD127low acting through direct cell to cell contact, suppressive cytokine secretion or through competition for growth factors linking (tethering),

c) CD8+ T regs functioning during the secondary and memory phases of the immune response,

d) TCRγδ+ T cells showing suppressive function towards TCRαβ+ T cells, antigen presenting cells and granulocytes.

Myeloid derived suppressor cells (MDSCs) are a morphologically, phenotypically and functionally heterogeneous population of immature cells of myeloid origin produced in the bone marrow in inflammatory conditions, including some types of cancer in order to protect the body from the consequences. For educational purposes the cells are ranked into monocytic, granulocytic and immature MDSCs. They have many common and many different characteristics.

MDSCs favor the tumor increase and development through Tregs induction in the tumor microenvironment, through suppressing T cells migration and survival. In addition, MDSCs promote neoangiogenesis or metastasis through soluble factors secretion. [17].

FUNCTIONAL ASSAYS BY FLOW CYTOMETRY

Flow cytometry not only is a critical component in the identification and quantification of immune cells, but it has also emerged as a well established method to evaluate cellular functions that are critical to the activation and regulation of immune responses [18]. A great advantage of functional assays performed by flow cytometry is that they can be combined with simultaneous immunophenotyping. When used in mixed cell populations, as peripheral blood, they allow therefore to assign the function studied to a specific cell type.

Cell proliferation

We started illustrating different methods that can be used to assess cell proliferation by flow cytometry, including assessment of the cell cycle phases, assessment of DNA synthesis, the carboxyfluorescein diacetate succinimidyl ester (CFSE) dilution assay, and measurement of proliferation proteins. Pros and cons of each single approach were discussed, and exemplificative analyses were presented [19-21].

Cell apoptosis

We then outlined the main strategies used to assess cell apoptosis by flow cytometry, based on the assessment of mitochondrial transmembrane potential, activation of caspases, DNA fragmentation, and plasma membrane alterations [22].

We provided detailed information on the most widely used strategy, based on Annexin V staining combined with plasma membrane permeability markers. We illustrated the experimental protocol, and presented exemplificative analyses showing that with this approach a distinction can be made between live, apoptotic, and late apoptotic-necrotic cells [23].

Cytokine production

Finally, we provided an overview of the methods that can be used to assess cytokine production, illustrating the flow cytometric approaches in the general scenario. We illustrated in detail the experimental protocol for intracellular cytokine detection, lingering on the most critical steps of the procedure. Exemplificative analyses were showed and discussed [24]. Other flow cytometric methods to assess cytokine production, including the secretion assay and particle-based multiplexed assays, were also illustrated. Tips and tricks relative to all the functional assays illustrated in this session were discussed.

Phagocytosis

Innate immunity is the body's first line of defense, immediate and non-specific, with no memory. The basic mechanism of non-specific immunity includes the ability to recognize, intake, integrate and intracellular kill "foreign" elements from the "professional" phagocytes of the immune system which are the monocytes/macrophages, the dendritic cells, and the neutrophils.

Phagocytes express on their surface Pattern Recognition Receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs) as well as high affinity receptors (Fc receptors) that recognize molecules such as "complement" and antibodies.

The presentation included a detailed explanation of a phagocytosis assay that could be performed in an immunology laboratory. Briefly, it was described how the samples are incubated under the appropriate conditions and the opsonized E. coli labeled with fluorescein bacteria are killed by the phagocytes in the sample. Quantitative and qualitative measurement of the intensity of fluorescent bacteria phagocytosed corresponds to quantitative measurement of phagocytic capacity.

Oxidative burst

For the oxidative burst it was explained how this method is applied for the quantitative measurement of oxygen free radicals produced during the respiratory burst of the cell. Dihydrohodamine is a membrane-permeable colorless substance which, when oxidized by the H2O2 released during the final phase of phagocytosis, converts to fluorescent red pigment rhodamine 1,2,3. The percentage of cells that fluoresce with rhodamine 1,2,3 (R 1,2,3) and therefore have a sufficient oxidation mechanism is measured by flow cytometry.

For both functional tests, it was made sure that certain preanalytical and analytical pit falls were explained as well as the usefulness of these assays as diagnostic tools in an immunology laboratory.

Cytotoxicity and basophil degradation

Secretory lysosome (SL) generation and their exocytosis are complex and articulated processes that need to be finely understood in order to choose the proper protocol when testing a cell population functionality. In this course, after a biomolecular and functional characterization of how, upon different stimulations, degranulation takes place in different cell types, we explained the most used degranulation assays and cytotoxicity tests together with tip and tricks useful to obtain clear and reproducible results. Since degranulation tests are fundamental not only in the diagnosis of some rare primary immune deficiencies like Chediak-Higashi and Griscelli Syndrome but also in the evaluation of allergies, we went deep in detail in the description of basophil activation test (BAT). A fine description of all the different phenotypic and activation markers [25] together with and extended explanation on which should be used in any different occasion was given [26]. Moreover, various usable protocols and the reasons for choosing one or another where discussed together with the students in a friendly and, we hope, constructive atmosphere.

Flow x-match

A very important and old flow cytometry application is crossmatch (XM) for organ (usually renal) transplantation. For this purpose, the donor's cells are incubated with recipient's serum as well as a positive and a negative control. Monoclonal antibodies for T (CD3) and B (CD19) cells are added together with anti-human IgG, in order to determine the donor HLA-specific IgG antibodies in the recipient's serum. The ratio of median fluorescence intensity (MFI) of the IGG expression after the incubation of the potential recipient's serum with the donor's cells to the MFI of IgG after the incubation of a negative serum with the donor's cells is one of the evaluation methods of the result.

It is very important to establish the cut-off value for the distinction between positive and negative crossmatch by performing at least 30 unrelated crossmatches and establishing the mean value plus 2 SDs.

The advantages of flow crossmatch are the following:

a) it is less subjective, quantitative,

b) it detects low titer antibodies (10-250 times more sensitive than CDC-XM),

c) it detects complement activating and non-activating abs (IgG1, IgG2, IgG3 IgG4, IgA, IgM).

The disadvantages are the following:

a) it is not a functional method,

b) Abs detected do not reflect their capacity to activate C. Do they harm the graft in vivo?

c) there are false positive results especially regarding B cells.

Although the positive CDC-XM is an absolute contraindication for transplantation, positive T cell FC-XM is a relative one [27].

FLOW CYTOMETRY IN HEALTH AND DISEASE

Flow cytometry in primary immunodeficiencies

The introduction to primary immunodeficiencies (PIDs) was made by briefly referring to the basic steps of the immune response and by linking their defects to the basic PID groups. We next described the algorithms which are most broadly used for PID diagnosis, starting from basic assays, passing through the more specific immunophenotypic studies and culminating to more elaborate functional tests. Throughout the lecture we tried to raise awareness of PIDs, to deal with some misconceptions concerning them and, therefore, to convince the participants that PIDs are not as rare as widely thought and that a flow cytometrist should be able to recognize, or at least suspect, the basic ones [28, 29, 30].

A brief reference to the follow up of the immune reconstitution post Hematopoietic Stem Cell Transplantation (HSCT) was made during a "surprise" 30 minutes course. The basic principles of HSCT and immune reconstitution were described, with an emphasis on antigen (mostly virus) specific T cells. A more thorough insight to this vast topic would be welcome in future courses.

Bronchoalveolar lavage (BAL)

Bronchoalveolar lavage (BAL) derives from a minimally invasive bronchoscopic technique. Specifically, by inserting the bronchoscope into the appropriate bronchus (the final bronchioles and lung alveoli), normal saline is gradually infused and aspirated. This way, the aspirate is used for the isolation of cells, inhalation particles, infectious agents and soluble non-cellular components. Furthermore a more representative picture of inflammatory and immunological processes at the alveolar level is achieved.

BAL is a diagnostic screening test of choice for sarcoidosis. As a complementary assay, is used for the diagnosis of various lung diseases, such as infections, interstitial diseases and malignancies. As a research tool is used to investigate the immunopathogenicity of lung diseases by contributing to the understanding of the immune and inflammatory mechanisms that prevail in various lung diseases. The BAL study includes macroscopic examination (appearance of the fluid) pathogen detection, cell morphology and immunophenotype.

Thus the presentation summarized the technique the procedure and the possible pitfalls that could occur during the process of BAL

Circulating tumor cells (CTCs)

Circulating tumor cells (CTCs) were first identified in 1869 by Ashworth in the blood of a man with metastatic cancer [31]. CTCs travel through the bloodstream or the lymphatic system and acquire the capacity to colonize in distant organs and finally establish a tumor metastasis [32]. CTCs can be found in the blood of patients as single cells or in groups of two or more adjacent CTCs termed as CTC clusters [33]. The ability of circulating tumor cells (CTCs) to form clusters has been linked to increased metastatic potential [34, 35].

The presence of CTC in the blood stream represents a rare event. However, despite the recent technical advancements, their isolation and detection remain a big challenge [36]. So far, the CellSearch system, is the only FDA-cleared semi-automated system for the isolation and enumeration of CTCs of epithelial origin [37]. CTC status can serve as an indicator to monitor the effectiveness of treatments and guide subsequent therapies [38, 39]. A recent meta-analysis conducted in early breast cancer patients treated by NCT, showed that CTC count

is an independent and quantitative prognostic factor [40].

CTC is a "liquid biopsy" approach for real time monitoring of cancer patients and assessment of treatment efficacy [41]. Molecular analysis of CTC provides significant insights into tumor heterogeneity, mechanisms of metastasis, tumor evolution and treatment resistance [42, 43]. Tumor specific biomarkers based on comprehensive characterization of CTCs could guide clinicians about the decision to prescribe targeted therapies to cancer patients [44, 45]. Using molecular assays, a variety of molecular markers such as multiple gene expression and DNA methylation markers have been detected and quantified in CTCs in various cancer types [46, 47].

In metastatic castration resistant prostate cancer (mCRPC) androgen-receptor splice variant 7 (AR-V7) is a highly promising liquid biopsy predictive biomarker showing primary or acquired resistance to novel androgen receptor signaling inhibitors [48, 49]. A novel multiplex RT-qPCR assay for the simultaneous detection of the androgen receptor (AR) and its splice variants AR-V7 and AR-567es, was recently developed and evaluated in circulating tumor cells (CTCs) and paired plasma-derived extracellular vesicles in mCRPC patients, showing distinct molecular patterns [50].

Checkpoint inhibitor-based immunotherapies have achieved impressive success in the treatment of different cancer types [51]. Abnormally high PD-L1 expression on tumor cells mediates tumor immune escape, and the development of anti-PD-1/PD-L1 antibodies has recently become a hot topic in cancer immunotherapy [52]. PD-L1 is frequently expressed on metastatic cells circulating in the blood of hormone receptor-positive, HER2-negative breast cancer patients [53]. It has been also reported, that PD-L1 over-expression in EpCAM(+) CTC fraction of HNSCC patients provide important prognostic information and represents a significant dynamic liquid biopsy biomarker since may evolve during treatment [44].

DNA methylation is an epigenetic mechanism that cells use to control gene expression [54]. Epigenetic modifications are very important in cancer development, since usually occur at an early stage [55]. The epigenetic silencing of key tumor suppressors and metastasis suppressors has been detected in CTCs by using Methylation specific PCR (MSP) for cystatin M (CST6) [56], Breast Cancer Metastasis Suppressor-1 (BRMS1) [57] and SRY-box containing gene 17 (SOX17) gene promoters. ESR1 epigenetic silencing potentially affects response to endocrine treatment. Mastoraki et.al. have shown that ESR1 methylation in CTCs is strongly associated with lack of response to everolimus/exemestane regimen showing the potential of ESR1 methylation as a liquid biopsy-based biomarker for endocrine treatment efficacy [47].

Immunomonitoring during treatment with biological drugs

Biological drug is a substance that is made from a living organism or its products and is used in the prevention, diagnosis, or treatment of cancer and other diseases. Biological drugs include antibodies, interleukins, and vaccines. Also called biologic agent and biological agent. Examples are:

- Immune check point inhibitors (PD-1, PD-L1, and CTLA-4 targets);
- Immune Cell Therapy (also called Adoptive Cell Therapy or Adoptive Immunotherapy) (TILs, CAR T cells);
- Therapeutic antibodies;
- Immune-Modulating Agents;
- Therapeutic Vaccines.

Regarding Monoclonal antibodies treatment, the cytometrist's tasks are to make a baseline assessment of the relevant antigen on target cells, to set the appropriate reagent protocol to assess monoclonal antibody efficacy, to set protocols to distinguish cell disappearance from antigen modulation, to check for adverse effects of the biological drugs (emergence of malignant clones, disappearance of other types of cells), and to assess reappearance of target cells [58].

CONCLUDING REMARKS

The School was attended by students from Italy, Greece, Czech Republic and Russia. All students greatly appreciated the organization of the course, the quality of all presentations, and the friendly and inclusive atmosphere of the School that offered a nice opportunity to learn and discuss technical and biological issues of the treated themes. All students reported that the Kos School was a very useful experience for their work needs.

The enthusiastic comments received by students indicated that the School was an effective tool for professionals working in cytometry immunology labs in Europe and throughout the world. It should be noted that one of the students is currently doing a one month experience in the research lab of one of the teachers to improve her cytometric and cell sorting skills

Proud of the obtained results, ESCCA will repeat the experience, taking into account important suggestions received by the attendants. Therefore, we are happy to announce that the second edition of the ESCCA Summer School in Flow Cytometry for Immunology will be held in Kos on June 3-7, 2020 (see Table 2, on the following page).

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Table 2 ESCCA Summer School in Flow Cytometry for Immunology (June 3-7, 2020 – Kos Island, Greece)

	6/3/2020	6/4/2020	6/5/2020	6/6/2020	6/7/2020
	Wednesday	Thursday	Friday	Saturday	Sunday
9.00-10.30		**Immunophenotyping in immunology:** Dendritic cells and other myeloid cells Silvia Della Bella (IT)	**Flow cytometry in health and disease:** Distribution of immune cells throughout life Perez Martin (SP)	**Flow cytometry in health and disease:** Flow cytometry in primary immunodeficiencies Marianna Tzanoudaki (GR)	**Functional assays by flow cytometry:** cytotoxicity, basophil degranulation Genny Del Zotto (IT)
10.30-12.00		**Immunophenotyping in immunology:** Suppressor cells Katherina Psarra (GR)	**Functional assays by flow cytometry:** cell proliferation, apoptosis, cytokine production Silvia Della Bella (IT)	**Flow cytometry in health and disease:** Immunomonitoring during treatment with biological drugs Katherina Psarra (GR)	**Concluding remarks**
12.00-13.00		**Presentation and discussion of data from attendees**	**Presentation and discussion of data from attendees**	**Presentation and discussion of data from attendees**	
13.00-14.00		free lunch	free lunch	free lunch	
14.00-15.30	**Immunophenotyping in immunology:** Fluorochrome choices for multi-color flow cytometry Perez Martin (SP)	**Immunophenotyping in immunology:** NK cells and innate lymphoid cells Genny Del Zotto (IT)		**Immunophenotyping in immunology:** Analytical tools for high-dimensional flow cytometry data Silvia Della Bella (IT)	
15.30-17.00	**Immunophenotyping in immunology:** T cells Katherina Psarra Alexandra Fleva (GR)	**Functional assays by flow cytometry:** phagocytosis, oxidative burst, autophagy Alexandra Fleva (GR)	**visit to Asklipieio**	**Functional assays by flow cytometry:** microvescicles Genny Del Zotto (IT)	
17.00-18.30	**Immunophenotyping in immunology:** B cells Marianna Tzanoudaki (GR)	**Flow cytometry in health and disease:** Circulating tumor cells Areti Strati (GR)		**Functional assays by flow cytometry:** Phosphoflow in immunology testing	

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