Infection and Immunity: Novel approaches of anti-tumor vaccination based on optimal stimulation and/or rescue of immune effector cells.

PIs: Roberto ACCOLLA, MD, and Greta FORLANI, PhD roberto.accolla@uninsubria.it

Summary program

Although recent therapeutic approaches have revitalized the enthusiasm of the immunological way to combat cancer, still the comprehension of immunity against tumors is largely incomplete. Due to their specific function, CD8+ T cells with cytolytic activity (CTL) have attracted the attention of most investigators because CTL are considered the main effectors against tumor cells. Nevertheless, CTL activity and persistence is largely dependent on the action of CD4+ T helper cells (TH). Thus establishment of tumor-specific TH cell response is key to the optimal response against cancer. CD4+ TH lymphocytes are conventionally primed and activated against antigens, including TAAs, by professional antigen presenting cells (APCs). Priming is mainly induced by dendritic cells (DCs) and less efficiently by macrophages, that present antigens via their cell surface MHC class II molecules.

Our research approach is based on rendering tumor cells MHC class II-positive and thus potential APCs for their own tumor antigens, by introducing the gene expressing the MHC class II transactivator (also designed CIITA), discovered in our laboratory. Previous results from our laboratory have demonstrated that effective rejection or significant growth retardation of the CIITA-driven MHC-II-positive tumor cells of distinct histological origins was accomplished (1). Moreover, successfully "vaccinated" mice were shown to be protected from challenge with the MHC-II negative parental tumor.

Can these experimental observations be integrated in a strategy applicable to human cancer? The straightforward conclusion from the above studies is the evidence of the strong immunogenicity of the MHC-II-tumor peptide complexes present on CIITA-positive tumor cells (2). Based on this evidence we would like to finally establish the potential of CIITA-tumors to act as original priming cells for naïve CD4+ TH cells (3) and, more importantly, from the translational aspect of our research, to construct an innovative tumor vaccine against several human carcinomas and the central nervous system (CNS) deadly glioblastoma tumor by purifying MHC-II-bound tumor peptides isolated from CIITA-modified tumor cells (TUMAPs) following a methodological purification strategy already described. The reason to focus particularly on glioblastoma is motivated by the increasing incidence, severity and lack of resolutive therapeutic tools against this tumor. Indeed, glioblastoma is the most common primary CNS malignancy accounting for 70% of all new CNS cancers.

Our approach will proceed stepwise, first by transfecting human tumor cells in vitro with CIITA, assessing the expression of MHC class II cell surface molecules, subsequently to isolate stable CIITA transfectants and finally proceed to the purification of MHC class II cell surface molecules and their bound peptides. Among those, through mass spectrometry, we will identify the sequence of those peptides expressed only on tumor cells and not on normal cells and proceed, by collaborating with other centers and particularly with Dr. Bassani-Sternberg at the Ludwig Institute for Cancer Research in

Lausanne, to the synthesis of the most tumor-specific and immunogenic peptides to be part of a suitable anti-tumor vaccine cocktail.

It is our belief that such a strategy will allow the display and identification of a much broader as well as more representative array of tumor peptides compared to those that professional APCs can display, due to their intrinsic limitation to process and present peptides derived only from exogenously engulfed, phagocytosed material.

The study of anti-tumor immune response in animal models has paved the way not only to increase our knowledge on basic mechanism of antigen presentation and triggering of CD4+ TH cells but also to apply the acquired knowledge for testing innovative strategies of anti-tumor vaccination in clinical setting.

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Title of the Project: Prostate cancer: disentangling the relationships within the tumour microenvironment to better model and target tumour progression.

Background. Affecting 1 man out of 7 in industrialized countries, prostate cancer (PCa) is one of the most common tumours in men over 60. At diagnosis, 90% of prostate cancers are confined to the organ. Since it is almost impossible to predict the pathological steps that lead to tumour aggressiveness, patients are often treated with partial or radical prostatectomy and/or anti-androgen

therapy. However, of the over 600.000 newly diagnosed PCa in the European Union and US every year, one third will progress to the metastatic stage of the disease for which no effective treatments are actually available. It is clearly emerging that the type of genetic and epigenetic alterations driving malignant transformation in the prostate epithelial cells (intrinsic factors) can predict much better than histology tumour behaviour and sometimes the response to specific therapies. Lethality of PCa is uniquely associated with the metastatic progression of the disease. We generated a genetic in vitro platform modelling the natural history of prostate tumourigenesis. For this purpose, the immortalized human epithelial prostate cell line RWPE-1 has been engineered to generate a 2D model of PCa with a panel of doxycycline-based inducible vectors to mimic:

1. ERG over-expression, a very early genomic event in prostate tumourigenesis affecting almost 50% of all PCa patients. ERG is not oncogenic per se, yet it sustains tumour progression when combined with a

transformation event such as PTEN dysfunction.

2. ERG over-expression in combination with partial PTEN downregulation.

3. ERG over-expression in combination with massive PTEN downregulation.

Hypothesis. We hypothesise that DNA methylation changes are required for ERG over-expression and PTEN down-regulation during PCa progression. Aims. The overall objective of the project is the identification of the transcription and DNA methylation profiles required for tumour reprogramming by ERG and PTEN modulation in 2D models of in vitro PCa model. Experimental design. To isolate differentially expressed transcripts required for tumour reprogramming in the 2D model that are dependent or independent from DNA methylation changes, we will or not knockdown DNMT-1, -3A, -3B, the three TETs prior to ERG and PTEN modulation, and the resulting phenotypes will be analysed for tumour and invasive parameters. For each experimental condition and relative controls, Illumina Infinium CytoSNP-850K BeadChip and RNA-seq for coding and non coding sequences will be performed. The combined differential analysis of the methylation profiles and of the RNA-seq data between each sample, will allow identifying coding and non coding transcripts dependent or not on DNA methylation changes that are induced or repressed by ERG and PTEN modulation and in response to the secretome signalling and that might be required for their action during tumour progression (driver transcripts).

To validate the driving role of those identified transcripts, knock down of the up-regulated or over-expression of the down-regulated transcripts (both coding and non-coding) will be performed prior to ERG and PTEN modulation in the 2D and 3D models, and the resulting phenotypes analysed Financing

The project is financed by EPIGEN – Progetto Bandiera with 400K€ in three years.

External collaborators

Prof. Andrea Lunardi, University of Trento, Trento

Prof. Valeria Poli, University of Turin, Turin

Prof. Licio Collavin, University of Trieste, Trieste

Prof. Alessandro Weisz, University of Salerno, Salerno

The PhD candidate will perform his/her PhD thesis in the lab of General Pathology at the University of Insubria and in collaboration with Prof. Alessandro Weisz at the University of Salerno for the -Omics experiments. Bibliography

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Physiology: Transmembrane protein as drug target and tools for drug delivery.

PI: Elena BOSSI, elena.bossi@uninsubria.it

Transmembrane proteins are the tools of the cell to exchange material and information. Channels, Receptors, and Transporters are the target of thousands of old and new molecules. The solute carrier proteins (SLCs) in particular SLC6, SLC11 and SLC15 are the main research focus of the laboratory of cellular and molecular physiology (LFCM). These are transporters protein that couple ions electrochemical gradient to the uphill transport of ions or organic substrates, they can be studied by many different approaches. In LFCM these proteins are heterologously expressed in Xenopus Laevis oocytes or in cell lines and characterized by biophysical (Electrophysiology), immunochemical (Immunocytochemistry, single oocyte chemiluminescence) or fluorimetric tools (fluorescence probe and protein). Molecular biology is the main technique used to prepare wild type, chimeric and mutated cDNAs for heterologous expression.

Cardiology – PI: Prof. Roberto De Ponti

Cardiovascular diseases: Evaluation of procedure parameters and clinical outcomes in populations of patients undergoing catheter or surgical ablation for complex atrial tachyarrhythmias.

Background: Some patients presents with complex supraventricular arrhythmias that require lengthy and more complex ablation procedure with still variable and in some cases suboptimal acute and mid- or long-term results. Complex supraventricular tachyarrhythmias represent a quite wide spectrum of cardiac arrhythmias. In fact, atrial tachycardias arising in humans after either a variety of cardiac surgical procedures or catheter ablation for complex atrial arrhythmias do represent a challenging clinical scenario in cardiac electrophysiology. Intra-atrial re-entrant tachycardias or post-incisional atrial arrhythmias arising after heart surgery for congenital heart disease1, mitral valve replacement or repair2, and surgical ablation of atrial fibrillation (i.e. Cox-Maze procedures)3 have been previously described. Moreover, pulmonary vein isolation for paroxysmal and persistent atrial fibrillation may lead to these complex atrial arrhythmias, especially when the ablation lines performed in the left atrium are not complete4. In fact, atrial remodelling caused by scarring due to either post-surgical incisions and cannulation sites or extensive lesions performed during catheter ablation represent the main pathophysiological mechanism underpinning these complex atrial macro-reentrant tachycardias. These iatrogenic scars may create areas of slow conduction that may provide a substrate for a re-entrant mechanism, triggering and maintaining these complex arrhythmias2. Therefore, the epidemiology of these arrhythmias depends on both the anatomical substrate, type of surgery or catheter ablation procedure, and the burden of atrial scarring in these patients. It has been reported a great variety in the prevalence of intra-atrial re-entrant tachycardias in patients affected from congenital heart disease (from 2% to 50%)1. On the other hand, incidence of atrial tachycardias after radio-frequency catheter ablation of atrial fibrillation varies depending on the extent of ablation in the left atrium with a reported incidence varying from 1% to 20% in these patients4. Palpitations, dizziness, dyspnea or fatigue do represent the usual clinical presentation in these patients, but in some cases it may be more dramatic, leading to haemodynamic instability and potentially death. In these latter cases, the onset of complex arrhythmias may herald declining intra-cardiac haemodynamic and could provide a marker of risk of severe structural heart disease1. Generally, anti-arrhythmic drugs do not fully control the symptoms and trans-catheter ablation may offer a permanent solution in these patients. Since their introduction in the 1998, three-dimensional electro-anatomic mapping systems may further help the cardiac electrophysiologist in these tasks by providing an accurate intracardiac reconstruction of the arrhythmia circuit and performing precise radiofrequency ablation of the arrhythmic substrate (mid-diastolic isthmus)5. Currently, they represent a very well-established technology with a clear impact on procedure parameters6. Although these systems have been used for at least two decades, data regarding the outcome and safety profile of catheter ablation of complex atrial arrhythmias using the three-dimensional mapping systems in a variety of post-surgical and catheter ablation procedures are scarce and data on large cohorts of patients are lacking.

Objective: the aim of this research project is to evaluate the procedure parameters and the clinical outcomes during follow-up in patients undergoing catheter ablation or surgical ablation for complex atrial tachyarrhythmias mentioned above, such as atrial fibrillation, atrial macro-reentrant tachycardias, or atypical atrial flutters arising after an index catheter or surgical ablation. For the different types of targeted arrhythmias, the overall acute success, overall complications, and long-term clinical outcomes of catheter ablation of these complex arrhythmias will be investigated with further sub-analysis in specific subgroups to identify also the subgroups of patients who may benefit more from the

procedure, the optimal timing of the procedure, or the best procedure methodology on a risk/benefit ratio.

Research methodology: deep revision of the literature in the form of a meta-analysis, single-center case series of consecutive patients or multicentre collaborative surveys will be performed to address the aim of this research project.

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Neuroscience

Adaptive changes of the intestinal neuromuscular function to intestinal ischemia/reperfusion injury: role of hyaluronan

Tutor: Dr Cristina Giaroni

Intestinal ischemia/reperfusion (I/R) injury, caused by an insufficient supply of blood flow to all or part of the gastrointestinal (GI) tract, has severe consequences on different cell types constituting the enteric microenvironment including enterocytes, smooth muscle cells, enteric glial cells and neurons. Myenteric neurons are especially sensitive, and can be irreversibly damaged. From a functional viewpoint, I/R-induced neuronal loss entails hindered food digestion and slowing of GI transit. In recent years, we have focused on hyluronan (HA), an unbranched glycosaminoglycan (GAG) component of the extracellular matrix, as a new molecular player involved in neuroadaptive changes of enteric neuronal circuitries in the inflamed gut. In particular, we have demonstrated that in the rat colon myenteric neurons produce HA, mainly via the HA synthesizing enzyme (HAS2), which retains a homeostatic role by contributing to the formation of an extracellular matrix basal membrane enveloping the surface of myenteric ganglia as well as a perineuronal net surrounding myenteric neurons. After an experimentally-induced colitis, however, this well organized HA structure, within myenteric ganglia, is highly altered and may participate to myenteric neuron derangement underlying changes in motor function. Since chronic inflammatory bowel diseases often include episodes of ischemia, in this study we aim to evaluate possible changes in HA homeostasis in myenteric ganglia after an in vivo-induced I/R damage. To this end the following approaches will be carried out in an established model of an in vivo ischemia/reperfusion injury in rat small intestine, in the absence and presence of an inhibitor of HA synthesis, 4-methylumbelliferone:

The project will comprise the following approaches:

-**Functional studies**:

-in vivo measurement of the efficiency of the intestinal transit;

-in vitro organ bath studies in order to evaluate the excitatory response to electrical field or to pharmacological stimulation, the inhibitory non-adrenergic non-cholinergic (NANC) response to electrical field stimulation.

-Biomolecular studies:

-q-RT-PCR and western blotting experiments to evaluate the expression levels of HA synthesizing enzymes (HAS1 and HAS2) and of HA receptor, CD44 in the longitudinal muscle myenteric plexus, submucosal and mucosal preparations.

-Immunohistochemical studies:

-to evaluate the distribution of fluorescence of a FITC-labelled HA binding protein in intestinal cross-sections and in longitudinal muscle myenteric plexus preparations. Immunohistochemical evaluation of the distribution of HAS1 and HAS2 in longitudinal muscle myenteric plexus preparations.Evaluation of the chemical coding of HA expressing myenteric neurons by co-staining with different main enteric neurotransmitters

-Biochemical studies:

ELISA assay in order to measure intestinal HA levels

Neuroscience:

Mitochondrial dysfunction in the pathogenesis of Parkinson's disease.

Mauro Fasano

Tiziana Alberio

Parkinson's disease (PD) is a multifactorial disorder whose etiology is not completely understood. Strong evidences suggest that mitochondrial impairment and altered mitochondrial disposal play a key role in the development of this pathology. This association has been demonstrated in both genetic and sporadic forms of the disease.

The project aims at clarifying the mitochondrial landscape and molecular mechanisms underlying Parkinson's disease, with a specific focus on investigating the consequences of impaired clearance of dysfunctional mitochondria.

As cellular models, we will use both toxin-induced models of PD in immortalized cell lines, thus highlighting the importance of environmental factors in the onset of this pathology and patients' fibroblasts. In particular, we will focus our attention on mitochondrial dynamics, mitochondrial biogenesis, and mitophagy. By the use of standard biochemical assays (e.g., Western blotting, Immunofluorescence), we will investigate main molecular factors involved in mitochondrial dynamics. Moreover, we will exploit proteomics, a global and unbiased approach suitable to unravel alterations of the molecular pathways in multifactorial diseases, thus contributing to the Biology and Disease pillar of the mitochondrial human proteome project (mt-HPP). By integrating biochemical assays and proteomics results, a bioinformatics model of molecular mechanisms underlying mitochondrial impairment in PD will be generated. Proteins mainly involved in mitochondrial impairment will be mapped on the functional mitochondrial human proteome network. Moreover, mathematical dynamic models of the altered mitochondrion will be generated in collaboration with informaticians. A better understanding of the molecular factors will help to unravel new potential therapeutic targets in PD.

The PhD student involved in the project will choose, based on her/his skills and preferences, on which specific aspects concentrate the PhD thesis. She/he will be asked to design and perform experiments and critically interpret the results.

ONCOLOGY PI: Daniela Furlan (daniela.furlan@uninsubria.it)

Epigenetics and genetics cooperate in the development of sporadic colorectal cancers with defective mismatch repair

Background and preliminary data

Colorectal cancer (CRC) is one of the leading causes of mortality and morbidity in the world. About 15% of sporadic CRCs are deficient in DNA mismatch repair (MMR) and the hallmark of these tumors is a genome-wide hypermutability of short repetitive sequences that is called Microsatellite Instability (MSI). The pathogenesis of MSI CRCs is often non-classical because they arise from precursor lesions such as sessile serrated colorectal polyps rather than conventional adenomas.

For some time, it has been known that sporadic MSI CRCs represent a distinct genetic and clinicopathological entity [1] and they result from loss of MMR through epigenetic silencing of the MutL homolog 1 (MLH1) promoter by DNA methylation. The biological mechanisms underlying the methylation have not been investigated in detail until recently. Fang et al. [2] have demonstrated in cancer cell lines that BRAF oncogenic mutations mediate the CpG island methylation phenotype (CIMP) resulting in hypermethylation at MLH1 and other CIMP marker genes, via the transcriptional repressor MAFG4.

A major focus of the present project is on understanding the biological mechanisms underlying the development of sporadic MSI CRCs by exploring the role of common genetic single nucleotide variants (SNP) that were found to be associated with increased risk of CRCs.

In more details, our preliminary work in collaboration with Dr Annabelle Lewis (Wellcome Centre for Human Genetics at the University of Oxford) focused on the role of a CRC predisposition variant located in the MLH1 promoter, i.e. the SNP rs1800734 that lies between the transcription start site and the ATG codon in the 5' untranslated region of the gene. An association between rs1800734 and CRC risk has been shown in multiple candidate studies [3-6]. However, this strong association is limited to MSI CRCs, and is weak or absent in un-stratified data sets.

Our data support the hypothesis that MLH1 repression is the main mechanism by which rs1800734 confers cancer risk and demonstrate that the risk (A) allele leads to significantly higher MLH1 methylation levels and this strongly correlates with lower mRNA expression of the gene.

Transcription factors such as TFAP4, bind to rs1800734 region but with much weaker binding to the risk than the protective allele. Interestingly TFAP4 binding is absent on both alleles when promoter methylation is present (Thomas R, submitted).

ONCOLOGY PI: Daniela Furlan (daniela.furlan@uninsubria.it)

Specific aims

Aim 1: Genotyping of rs1800734 will be carried out in a consecutive series of 400 sporadic CRCs diagnosed at Ospedale di Circolo, ASST Settelaghi in Varese (Italy) in the last three years:

All these tumors were routinely evaluated for MMR immunohistochemical expression according to the project of Lombardy Region Oncologic network 4, work packaging 7. The frequency of MMR defective CRCs in our patient population was higher compared with data from literature (19.8% compared with 10-15%, respectively) and epigenetic silencing of MLH1 was observed in most of CRCs with defective mismatch repair (Chiaravalli AM, submitted).

This analysis will help to assess the allelic frequency of MLH1-93A in these patients compared with data reported in Caucasians, allowing to explore in our population, the role of this genetic variant in MSI CRC predisposition.

Aim 2: In the subset of sporadic MSI CRCs we will analyze the associations between rs1800734 alleles, MLH1 methylation levels, MLH1 mRNA expression, and BRAF mutation status.

This correlation analysis could help to verify a potential combined effect of somatic and germline factors in defining the specific biology of MSI CRCs.

Working hypothesis and translational implications Gene silencing is a well-known mechanism associated with sporadic carcinogenesis. However, the recent discovery of common genetic variants in cancer predisposition that may influence methylation acquisition in tumor suppressor genes, opens new perspectives to understand the interaction of epigenetic and genetic mechanisms underlying cancer.
We propose that rs1800734 genotyping may be useful to identify patients at risk for developing MSI CRC. Moreover, we hypothesize that the highest levels of MLH1 methylation may be observed when somatic activation of BRAF/MAFG pathway and rs1800734 risk allele (A) cooccur, suggesting a combined effect of somatic and germline factors in defining a specific subset of CRC with defective mismatch repair.

Collaborators

Dr. Annabelle Lewis, Cancer Gene Regulation Group, Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford, UK

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ONCOLOGY PI: Daniela Furlan (daniela.furlan@uninsubria.it)

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Project title: "Synaptic defects in CDKL5 disorder: identification and characterization of new targets for therapeutic intervention."
PI: Charlotte Kilstrup-Nielsen

Background: Mutations in the X-linked CDKL5 gene (cyclin-dependent kinase-like 5) cause a neurological disorder characterized by the early onset of drug-resistant epilepsy, intellectual disability, hypotonia, gross motor dysfunctions etc. The loss of CDKL5 impacts on various neuronal aspects such as morphology and synaptic functions, which are likely to underlie the observed epilepsy and cognitive defects in patients. The main interest of our laboratory is to identify the molecular network controlled by CDKL5 in neurons thus increasing the comprehension of the molecular basis of the observed phenotypes as well as the identification of novel druggable targets.

Project:

The focus of the present project is to characterize mitochondrial defects in cell and mouse models of CDKL5 disorder and the underlying molecular basis. The student will explore mitochondrial morphology, dynamics and functionality in murine Cdkl5-WT/KO neurons, brains and lymphocytes, and study the contribution of mitochondrial defects to Cdkl5-dependent morphological and synaptic defects. Moreover, validation of the data in patient derived peripheral, circulating and neuronal cells will disclose the relevance for the human pathology and possibly identify functional non-tissue specific biomarkers to monitor the progression of the disease.

Interested candidates should have a background in molecular and cellular biology and be willing to work with rodents.

Contact: c.kilstrup-nielsen@uninsubria.it

Selected publications.

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Impact of lymphatic drainage and extracellular tissue remodeling on cardiac tissue function.
PIs: Daniela NEGRINI and Andrea MORIONDO

The cardiac tissue is supplied by an extended network of lymphatic vessels dispersed in the sub-epicardial, myocardial and sub-endocardial areas and in atrioventricular and semilunar valves. As in the majority of body tissues, an efficient and normal lymphatic drainage guarantees the maintenance of a steady state interstitial fluid volume and solute concentration and returns macromolecular debris, leucocytes and cells to the blood stream. On the other hand, lymphatic saturation and/or failure may cause fluid accumulation and acute or chronic tissue edema, altered interstitial fluid solute concentration, deposition of tissue debris and acute and/or chronic inflammation. The result of lymph flow obstruction is an abnormal fibrous tissue deposition and remodeling, a condition that, in the heart, may lead to the development of several acute or chronic heart diseases, such as severe myocardial injury with subendocardial edema, intracellular edema, myofibrillar and mitochondrial degeneration. Two macromolecules may be particularly involved in tissue remodeling: hyaluronic acid (HA), the main anion hydrated macromolecule in the tissue and very sensitive to tissue water content and therefore lymphatic drainage, and collagen 1, which is the extracellular unsoluble macromolecule most overexpressed in inflammation. Of great importance from the clinical standpoint, it is the observation that lymphatic inefficiency and local lymphedema seem to be strictly associated with conduction disturbances and cardiac arrhythmias. Such an observation is particularly interesting on considering that lymph propulsion within large collecting lymphatics depends upon contraction waves triggered in lymphatic smooth muscle cells by spontaneous action potentials sustained by an inward current, carried by the so called hyperpolarization-activated cyclic nucleotide-gated (HCN) channels which belongs to the same channel family carrying the heart spontaneous sinoatrial pacemaker current.
Hence, the present research project aims at investigating the still largely unknown relationship between normal or altered heart frequency and cardiac lymphatic function. In particular we will focus on :
1. the interplay between external tissue forces and the intrinsic lymphatic contractility in sustaining and modulating cardiac lymph flow in normal and diseased heart, to establish whether the spontaneous lymphatics contractility matches and/or is modulated by the cardiac frequency. We hypothesize that cardiac lymph flow requires coordination of the cardiac and lymphatic pace-maker firings, likely through HCN channels;
2. the functional link between cardiac lymphatic impairment and disturbances of cardiac pacing. In fact, it is at present unknown whether impairment of the cardiac lymphatics induces matrix remodeling and, as a consequence, altered electrical disturbances of cardiac conductive fibers or, viceversa, if a primitive disturbance of the cardiac pacing eventually results in lymphatic inefficiency and, triggering a vicious cycle, altered matrix deposition and irreversible arrhythmia.
To pursue the above aims we will carry on:
A. functional studies:
• to determine to what extent modification of cardiac frequency in the normal heart may either improve or hinder cardiac lymph flow in sub-epicardial lymphatics
• to evaluate the pattern and amount of cardiac lymph flowB. morpho-functional studies:
• to verify and characterize the presence of smooth muscle cells in the wall of the cardiac lymphatic vessels
• to map the specific location of vessels with smooth muscle cells in the different cardiac areas (atria or ventricular walls, conduction tissue, valves)
• to relate cardiac lymphatic function to extracellular tissue structure, with particular focus on HA and collagen type 1
• to examine whether smooth muscle cells in cardiac lymphatics express HCN channels and characterize the specific HCN families, to be compared with the HCN channels expressed by the heart conduction system

The results of the present project might provide useful improvements of basic knowledge of the cardiac lymphatic function in healthy and diseased heart and a toll for the potential development of new therapeutic approaches.

ONCOLOGY

Project title: NEW MOLECULAR DIAGNOSTIC APPROACHES TO STUDY LEUKEMIC STEM CELLS AND THEIR ROLE IN THE PATHOGENESIS OF CHRONIC MIELOID LEUKEMIA

PI: Prof. Giovanni PORTA – giovanni.porta@uninsubria.it

La leucemia mieloide cronica (CML) rappresenta il 20% delle leucemie nell'adulto. La CML origina nel 95% dei casi da aberrazione cromosomica, una traslocazione reciproca bilanciata tra i cromosomi 9 e 22 (t(9;22)(q34:q11)) all'interno di una cellula staminale pluripotente, con Formazione del cromosoma Philadelphia (Ph).

La traslocazione ha come risultato la formazione del gene di fusione BCR-ABL1, la cui attività costitutiva promuove a proliferazione e la sopravvivenza cellulare, con resistenza all'apoptosi, l'aumento dell'invasività e la comparsa di metastasi.

Le metodiche classiche attualmente in uso per la diagnosi di CML sono la conta delle cellule del sangue con valutazione della loro morfologia, l'analisi citogenetica, mediante analisi del cariotipo e Fluorescent In Situ Hybridization (FISH); queste metodiche consentono la diretta individuazione del cromosoma Philadelphia nelle cellule leucemiche ma sono poco sensibilità.

La terapia di prima linea è costituita dall'Imatinib mesilato (Gleevec/Glivec, Novartis Pharma), uno specifico inibitore delle proteine tirosin-chinasiche, che porta ad una inibizione della proliferazione. Lo svantaggio di questo tipo di terapia è la necessità di dover assumere il farmaco a vita e la necessità di monitoraggio continuo per seguire la loro risposta al farmaco e controllare che non ci sia una ricaduta della malattia, mediante determinazione della malattia minima residua (MMR), ossia la percentuale di cellule leucemiche non eradicate dalla terapia.

gDNA-PCR, la nuova tecnica ad alta sensibilità per l'individuazione di cellule leucemiche nel paziente affetto da CML

La qRT-PCR è la tecnica più sensibile oggi disponibile per monitorare i livelli di mRNA di BCR-ABL1 dopo la diagnosi e l'inizio del trattamento. I risultati sono espressi come il rapporto tra il numero di trascritti di BCR-ABL1 e i trascritti di un gene controllo. Limiti di questa tecnica sono legati all'efficienza di estrazione, di retrotrascrizione ed alla qualità dell'RNA estratto. Inoltre, è stata riscontrata l'assenza di una correlazione tra i livelli di mRNA ed il numero di cellule leucemiche, in quanto si potrebbero avere cellule che trascrivono in quantità elevata, o cellule quiescenti. I risultati negativi sono, quindi, difficili da interpretare, in quanto l'assenza di trascritto chimerico potrebbe essere imputabile o ad un'effettiva eliminazione delle cellule leucemiche, oppure alla presenza di cellule leucemiche trascrizionalmente silenti. È pertanto fondamentale, di fronte a risultati negativi, riuscire a capire se il paziente sia effettivamente guarito e quindi si possa interrompere la terapia con Imatinib.

Attualmente però, la raccomandazione è quella di non interrompere la terapia, nonostante i considerevoli costi ed effetti collaterali negativi della chemio terapia.

Il nostro gruppo di ricerca ha messo a punto e validato una metodica innovativa, basata su una PCR quantitativa in tempo reale (quantitative real-time PCR, qRT-PCR) che amplifica la sequenza genomica della regione di rottura in BCR-ABL1. Ogni paziente affetto da LMC presenta un punto di rottura unico, con una sequenza di fusione specifica. Trovare il preciso punto di rottura porta all'individuazione di un marcatore tumorale paziente specifico che consente il monitoraggio della malattia minima residua durante la terapia. Sono stati sviluppati 16 saggi paziente-specifico di PCR quantitativa su DNA genomico basato

sul break-point di BCR-ABL1 con cui sono stati monitorati 16 pazienti affetti da CML in fase cronica e sotto trattamento con TKIs per 5-8 anni. È stata inoltre sviluppata una formula per calcolare il numero di cellule positive al cromosoma Philadelphia. Paragonando i nostri risultati con quelli ottenuti mediante tecniche standard, abbiamo dimostrato che la nostra metodica mostra la presenza di cellule positive al cromosoma Philadelphia in 7 dei campioni che non presentavano livelli misurabili di mRNA chimerico (Fig. 2).

Ad oggi sono stati selezionati, presso l'Ospedale Niguarda di Milano, 87 pazienti candidati all'interruzione della terapia con Imatinib e Nilotinib di cui è stata fatta la caratterizzazione molecolare del break-point genomico. Successivamente questo marcatore paziente-specifico verrà utilizzato per il monitoraggio della MMR nei follow-up.

La tecnica da noi utilizzata si basa su di un arricchimento della regione genomica d'interesse e un successivo "deep sequencing" per sequenziare il break-point a livello di singolo nucleotide. Una volta caratterizzato il punto di rottura paziente specifico la malattia minima residua potrà essere monitorata su campioni di sangue periferico nei follow-up successivi all'inizio della terapia.

Lo scopo della presente ricerca è quello di sviluppare un protocollo di stop-Imatinib italiano mediante monitoraggio del DNA genomico con metodica qRT-PCR. Ogni paziente avrà quindi un marcatore specifico caratteristico delle cellule leucemiche, che potrà essere utilizzato per assicurarsi dell'assenza di cellule Philadelphia positive prima dell'interruzione della terapia con Imatinib e Nilotinib.

Project title: **NEW MOLECULAR DIAGNOSTIC APPROACHES TO STUDY LEUKEMIC STEM CELLS AND THEIR ROLE IN THE PATHOGENESIS OF CHRONIC MIELOID LEUKEMIA**

Chronic Myeloid Leukemia (CML) represent the 20% of leukemia in adults. It originates in 95% of cases from chromosomal aberration, a balanced translocation between chromosomes 9 e 22 (t(9;22)(q34:q11)) in a pluripotent staminal cell, resulting in Philadelphia chromosome (Ph) formation.

The translocation results in the formation of the fusion gene BCR-ABL1, whose constitutive activity promotes proliferation and cellular survivial, due to apoptosis resistance, increased invasiveness and formation of metastasis.

Classical methods to diagnose CML are associated to blood cell counts, evaluating morphology, cytogenetic analysis throughout karyotype and Fluorescent In Situ Hybridization (FISH); these techniques allows the direct identification of Ph chromosome in leukemyc cells but they have low sensitivity.

First line therapy is represented by Imatinib mesylate (Gleevec/Glivec, Novartis Pharma), a specific tyrosine-kinase inhibitor, that lead to proliferation inhibition. The drawback of this therapy is due to lifetime assumption of this drug and continuous monitoring required to follow drug response and eventually identify CML relapse, by monitoring the Minimal Residual Disease (MRD), i.e. the percentage of leukemic cells not eradicated by the therapy.

dDNA-PCR, a new high-sensitivity technique to identify leukemic cells in CML affected patients

qRT-PCR is nowadays the most sensitive technique to monitor BCR-ABL1 mRNA after diagnosis and treatment initiation. Results are expressed as the ratio between BCR-ABL1 and a control gene transcripts.

Estraction efficiency, RNA quality and retrotranscription efficiency are the main limits associated with this technique. Moreover, there is no linear correlation between mRNA levels and the number of leukemic cells, i.e. it is possible to have higly transcribing or quiescent cells. Negative results (i.e. no detection of mRNA) are hard to be interpreted, because the absence of chimaeric transcript can be due to effective eradication of leukemic cells or to quiescent cells. For this reason, it is essential to have a way to clearly determin if the leukemia has been eradicated and Imatinib therapy can be stopped.

Currently reccomendations is not to interrupt the therapy, despite considerable costs and collateral effects of chemotherapy.

Our group has developed and vaalidated a new method, based on a quantitative real-time PCR (qRT-PCR) that amplifies the genomic sequence corresponding to the BCR-ABL1 breakpoint. Each CML patient has an unique breakpoint and an unique nucleotide sequence and the precise identification of it results in a tumoral, patient-specific marker that allows us to monitor of MRD during therapy.

We have characterized the breakpoint sequence of 16 chronic phase CML patient, developing patient-specific gDNA qRT-PCR assays used to analyze TKIs treated follow ups for 5-8 years.

A mathematical formula has also been developed to calculate the Ph^+ cells.

Analyzing in parallel our results with those obtained from the standard qRT-PCR on mRNA we demonstrated that our technique identifies the presence of leukemic cells in 7 samples that were negative on mRNA analysis.

Nowadays, 87 candidates to stop TKIs have been characterized on their genomic breakpoint. We will use this patient-specific markers to monitor MRD in follow up.

Our technique is based on a targeted enrichment of the genomic region involved in recombination, followed by "deep sequencing" and identification of the breakpoint at a nucleotide level.

Once obtained the breakpoint localization, MRD will be monitored on follow-up peripheral blood samples.

The aim of this research is to develop a stop-Imatinib protocol through gDNA qRT-PCR monitoring. Each patient will have a patient-specific molecular marker that detect uniquely leukemic cells and that can be used to direcly determine presence/absence of Ph^+ cells before stopping the therapy.

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Neuroscience:
Role of the endocannabinoid system in adolescent brain development.
PI Tiziana Rubino tiziana.rubino@uninsubria.it

Adolescence is a time of important neurobiological and behavioral changes, but is also the period in which several mental illnesses emerge, included psychosis, mood disorders, and substance abuse.
Among all drugs, Cannabis is the most widely abused by teens and several clinical data suggest the presence of a relationship between adolescent Cannabis abuse and the risk for developing psychiatric diseases later in life. Consistently, we have demonstrated that adolescent female rats treated with the psychoactive compound of Cannabis delta-9-tetrahydrocannabinol (THC), develop a depressive/psychotic-like phenotype in adulthood. Interestingly, we observed that only adolescent, but not adult, THC exposure leads to this phenotype, suggesting that adolescence represents a vulnerable period for the psychiatric consequences of THC exposure. However, the molecular underpinnings of this vulnerability remain to be established.
During adolescence, brain undergoes intensive processes of neuronal refinement, especially in cortical regions. Adolescent brain maturation involves a thinning of the gray matter (GM – it contains the cell bodies, dendrites and axon terminals of neurons) as result of synaptic pruning processes, through which "redundant" synapses overproduced in the early years of life are being eliminated; and an increase in white matter (WM – it is made of myelinated axons). Myelin improves neural transmission, contributing to the enhanced brain-regional connectivity and cognitive function that occur during adolescence. Thus, alterations in synaptic refinement as well as in myelination during this sensitive period could confer a vulnerability to psychiatric diseases.
In the brain, the Endocannabinoid System (ECS) is an important neuromodulatory system involved in synaptic plasticity regulation. So far, many works have addressed adolescent brain maturation, but the involvement of the ECS in adolescent brain refinement remains to be elucidated. Recently, we have demonstrated that adolescent THC exposure deeply changes neuronal refinement, altering the expression of proteins involved in synaptic plasticity and brain functionality. Moreover, our preliminary data show that adolescent THC alters the expression of MOG and MBP, two important markers of myelination. Thus, it is alleged that Cannabis consumption during adolescent brain maturation may alter the ECS functionality, interfering with normal brain development, and eventually resulting in a major vulnerability to mental illnesses.
On these bases, our proposal is to thoroughly investigate the role played by the EC signaling in processes occurring in the adolescent prefrontal cortex of female rats. To clearly depict each step of adolescent brain maturation, analyses will be performed every 5 day, from 28 to 75 PND focusing
on the events of synaptic pruning and myelination. Next, through the administration of specific modulators of the ECS, we will study the impact of this modulation on markers of plasticity and myelination. Specifically, we will administer AM251, a selective antagonist of CB1 receptor, the major cannabinoid receptor in the CNS; URB597, an inhibitor of the enzyme fatty acid amide hydrolase (FAAH, the enzyme that catalyzes the intracellular hydrolysis of the endocannabinoid anandamide "AEA"), JZL184, a selective inhibitor of monoacylglycerol lipase (MAGL, the enzyme that preferentially catabolizes the endocannabinoid 2-arachidonoyl glycerol "2-AG") and THC.
With this approach, we will be able to elucidate the role played by the specific components of the ECS (CB1R, AEA and 2-AG) during adolescent brain maturation. Moreover, we will also understand the impact of EC tone disruption in triggering brain vulnerability to psychiatric conditions.

New prognostic and predictive molecular and genetic markers in the personalized management of non-Hodgkin Lymphomas.

Silvia Uccella, MD, PhD, e-mail: silvia.uccella@uninsubria.it

BACKGROUND

Non-Hodgkin lymphomas encompass a variety of distinct entities, defined by a constellation of features: morphology, immunophenotype, molecular and genetic features, and clinical behavior. Each of these elements takes part in the diagnostic and prognostic definition of the single entities, influencing the personalized management of the patient.

For the last 25 years, there has been a dramatic progress in understanding the genetics of lymphoid malignancies. Recurrent cytogenetic and genetic abnormalities have been identified for many lymphoma subtypes and are currently used in the diagnostic and prognostic definition of non-Hodgkin lymphomas. In addition new high throughput technologies, such as next generation sequencing and novel digital molecular barcoding technology have allowed a greater insight in the wide heterogeneity of specific lymphoma subtypes, such as diffuse large B-cell lymphomas.

In the context of growing effort for improving the cure rate of non Hodgkin lymphomas and the patients' survival, the molecular and genetic characteristics of these disease have been the start point for a new era of personalized treatments. In fact, non-Hodgkin lymphomas' management has been historically based on polychemotherapeutic regimens, the efficacy of which is related to increasing doses, leading to increased toxicity, and less toxic and more specific therapeutic agents were needed. Targeted therapy in non-Hodgkin lymphomas is based on the use of monoclonal antibodies and of other molecules which interfere with subcellular pathways regulated by specific genes. For each of these drugs, the knowledge of the molecular profile and of the genetic alterations of lymphoma cells is crucial in predicting the therapeutic response. Ongoing research is focused on discovering new genetic, cytogenetic and molecular features of non-Hodgkin lymphomas, which can help to better understand the pathogenesis of each entity and to identify new therapeutic targets.

AIMS

The proposed research program is aimed to investigate new potential therapeutic targets in non-Hodgkin lymphomas using molecular analysis in combination with cytogenetic, genetic and molecular biology approach. In particular, the major objectives will be

1. Investigate the immune check point blockade (PD1/PDL1) in diffuse large B-cell lymphoma;
2. Characterize recurrent cytogenetic abnormalities in follicular lymphoma and diffuse large B-cell lymphoma.
3. Analyze the gene expression and mutational profile of DLBCL using advanced technologies based on next generation sequencing and NanoString® technologies

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