ISSN 1669-9106



MEDICINA (Buenos Aires) 2016; 76 (Supl. I): 12-70

## SAIC SATELLITE SYMPOSIUM

## SIGNAL TRANSDUCTION

# TARGETED INHIBITION OF ONCOGENIC DRIVERS IN GLIOMA AND BREAST CANCER CELLS: NEW INSIGHTS INTO THE MECHANISMS OF THE DEVELOPMENT OF DRUG RESISTANCE

**VIKTORIA VON MANSTEIN AND BERND GRONER**

*Georg-Speyer-Haus, Institute for Tumor Biology and Experimental Therapy, Paul-Ehrlich-Straße 42, 60596 Frankfurt/Main, Germany*

Tumor cell resistance to drug treatment severely limits the therapeutic success of treatment. Tumor cells, exposed to chemotherapeutic drugs, have developed intricate strategies to escape the cytotoxic effects and adapt to adverse conditions. The molecular mechanisms causing drug resistance can be based upon modifications of drug transport or metabolism, structural alterations of drug targets or the adaptation cellular signaling. An important component in the transformation of cells and the emergence of drug resistance is the activation of the transcription factor Stat3. The persistent, inappropriate activation of Stat3 causes the expression of target genes which promote tumor cell proliferation, survival, invasion and immune suppression; and it is instrumental in the process of the emergence of resistance to both conventional chemotherapeutic agents and novel targeted compounds. For these reasons, Stat3 inhibition is being pursued as a promising therapeutic strategy.

We investigated and compared the effects of the tyrosine kinase inhibitor canertinib and the down regulation of the transcription factor Stat3 on signaling pathways and the cellular phenotypes of Tu-2449 glioma and MDA-MB-468 breast cancer cells. Tu-2449 are glioma cells in which the v-Src and Bmx tyrosine kinases, and the downstream effectors Akt and Stat3, are persistently activated. MDA-MB-468 are triple negative breast cancer cells in which the EGFR functions as oncogenic driver and the downstream effectors, the Mek and Erk kinases, Akt and Stat3 are persistently activated. Exposure of Tu-2449 cells to canertinib inhibited the activation of the Bmx kinase and the subsequent phosphorylation of Stat3, but had no effect on the activation of v-Src.

A single dose of 5 μM canertinib caused a transient G1 arrest, whereas prolonged administration of canertinib proved cytotoxic. The down regulation of Stat3 by specific shRNA did not affect cell viability and normal cell cycle progression *in vitro*, but single cell infiltration and tumor growth were inhibited *in vivo*. We conclude that the cytotoxic effects of canertinib in Tu-2449 cells are not solely mediated by Stat3 inhibition, but probably require the simultaneous inhibition of the Bmx and Akt kinases. Canertinib treatment of MDA-MB-468 breast cancer cells caused inhibition of the EGFR, Erk1/2 and Mek1/2 kinases and of the downstream effectors Stat3 and Akt. In contrast to the glioma cells, the down regulation of Stat3 was sufficient to kill MDA-MB-468 cells.

Canertinib exposure of Tu-2449 cells rapidly caused the inhibition of the Bmx kinase and the deactivation of Stat3 and prolonged exposure of the cells to canertinib caused the death of the large majority of the cells. Only a few cells became drug resistant and survived in tight clusters. When the canertinib resistant cells were expanded and cultured at lower cell densities, they regained their sensitivity towards canertinib. We measured the extent of Stat3 activation as a function of cell density and found that higher cell densities accompanied by increased Stat3 activation and a higher expression of Stat3 target genes. We suggest that Stat3 induction through tight cell-cell interactions, most likely through the engagement of cadherins, can counteract the inhibitory effects exerted by canertinib on Bmx. Cell-cell interactions induced Stat3 and compensated for the suppression of Stat3 by canertinib, thus transiently protecting the cells from the cytotoxic effects of the inhibitor.

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## STUDY OF HORMONE REGULATION OF MITOCHONDRIAL FUSION/FISSION AS A PLATFORM FOR SUBCELLULAR COMPARTMENTALIZATION AND PROTEIN LOCALIZATION IN ENDOCRINE SYSTEMS

**CECILIA PODEROSO**

*INBIOMED-UBA-CONICET, Facultad de Medicina, Universidad de Buenos Aires, Argentina.*

The mitochondrion is a dynamic organelle that responds to fluctuations in cellular metabolic demand by changing its shape, number and even its intracellular distribution. These changes are the result of the balance between the activity of different processes such as fusion and fission, known as mitochondrial dynamics. Alterations in dynamics are involved in the evolution of many diseases, as neurodegenerative and immunological diseases, cancer, diabetes, etc. Mitochondria are tightly associated with the endoplasmic reticulum (ER) forming a subcellular domain per se, called mitochondria-associated membranes (MAM). Despite the importance of mitochondrial dynamics, little is known about its regulation in endocrine systems.

Steroid producing cells/tissues are very interesting models to study these mechanisms as steroid synthesis requires a very close interaction between membranes, through which cholesterol and its derivatives must transit.

Recently, we have shown that hormone stimulation increases mitochondrial fusion through the induction of Mitofusin 2 (Mfn2), a key fusion protein, in MA-10 Leydig cells. By immunofluorescence we observed a morphological shift of mitochondria from punctuated to elongated/tubular shape. Increased mitochondrial fusion is dependent of PKA activity, which mediates LH/hCG signaling pathway. We have also determined that fusion of mitochondria is necessary for the localization in mitochondria of a MAPK family member, ERK and also for the proper localization in MAM of an Acyl-CoA synthetase 4 (ACSL4), key enzyme in steroid production. By the downregulation of Mfn2 with an RNAi, we determined that Mfn2 plays a role in the regulation of the expression of the Steroidogenic Acute Regulatory protein (StAR), an essential protein for cholesterol transport to the mitochondria. We observed that phosphorylation by mitochondrial ERK of StAR promotes a lasting longer retention of this protein at the mitochondria to achieve a higher efficiency in cholesterol transfer. Our results suggest that mitochondrial fusion hormone stimulated can regulate the assembly of key proteins in mitochondria and concomitantly promote their functionality. Then, mitochondrial fusion can settle out the choreography used by cells to give specificity to the spatial and temporal response, in both physiological and pathological situations.

## (286) TNF ALPHA DOWN-REGULATION BY TRISTETRAPROLIN (TTP) IS RELEVANT FOR MOUSE LACTATION MAINTENANCE

**MARÍA VICTORIA GODDIO[1], ALBANA GATTELLI[1], LOURDES PÉREZ CUERVO[1], JOHANNA TOCCI[1], NANCY HYNES[2], ROBERTO MEISS[3], EDITH KORDON[1]**

*[1]IFIBYNE-UBA-CONICET, Buenos Aires, Argentina. [2]FMI, Basel, Switzerland. [3]Academia Nacional de Medicina, Buenos Aires, Argentina*

From pregnancy to lactation, lobulo-alveolar growth is followed by the complete differentiation of mammary epithelium, which allows the production and secretion of milk proteins. At weaning, a rapid switch from survival to death signaling occurs, leading to involution, which involves extensive remodeling and an innate immune response. TTP is a RNA-binding protein that leads to degradation of messenger RNA of pro-inflammatory cytokine and invasiveness-associated genes. We have previously shown that in the mouse mammary gland, TTP expression is induced during lactation and repressed after weaning. We have also determined, using conditional KO mice (TTP-MGKO), in which TTP expression is specifically down-regulated in the mammary secretory cells, that TTP prevents early cell death during lactation. Then, we found (by cytochemistry, immunohistochemistry, western blot and RT-qPCR analysis of mammary tissue) that TTP-MGKO glands show signs of involution at mid-lactation, like the presence of apoptotic epithelial cells, increased levels of cleft caspase-3, inflammatory cytokines (TNF$\alpha$, LIF and IL-6) and STAT3 phosphorylation, as well as a decrease in AKT phosphorylation. As TNF$\alpha$ mRNA is a main target for TTP destabilization activity, we speculated that the increase of this specific cytokine would play a primordial role in the development of the phenotype in the transgenic animals. To test this hypothesis, TTP-MGKO mice were inoculated with TNF$\alpha$ blocking antibody "Etanercept" during the first 15 days of lactation (n=4 in each group). This caused a significant reduction in mammary cell death, without modifying the high cytokine expression and Stat3 phosphorylation levels observed in the lactating TTP-MGKO placebo treated or untreated mice. These observations confirm that TTP significantly contributes to lactation maintenance, and shows that keeping TNF$\alpha$ levels low is fundamental for mammary cell survival during that period.

## (1022) AP-1 IS MODULATED BY FK506-BINDING PROTEIN 52

**MARÍA FERNANDA CAMISAY[1], SONIA DE LEO[1], VANINA FONTANA[1], DANIELA CONVERSO[1], MARIO GALIGNIANA[1,2], ALEJANDRA ERLEJMAN[1]**

*[1]Departamento de Química Biológica/ IQUIBICEN, FCEN, UBA and [2]IBYME, Buenos Aires, Argentina*

The FK506 binding protein 52 (FKBP52) is an Hsp90-binding protein with important regulatory functions on steroid receptor and others transcription factors such as NF-kappaB and p53. FKBP52 has two key domains: the tetratricopeptide repeat (TPR) domain, where Hsp90 binds, and the peptidylprolyl-isomerase (PPIase) domain, where its catalytic site is located. In view of the features shown by the Hsp90 cochaperone on the above-mentioned factors, we hypothesized that FKBP52 could also regulate the activity of AP-1 (activator protein 1). Transcriptional activity of AP-1 was evaluated by luciferase assays in HEK293T cells co-transfected with p-AP1-Luc and either expression vector: pCI-neo-FKBP52wt, pCI-neo-FKBP52K354A (a point mutant in the TPR domain, which does not bind Hsp90), or one of the PPIase domain mutants, pCI-neo-FKBP52F67Y or pCI-neo-FKBPP52F130Y. When cells were stimulated with 100 ng/ml PMA, AP-1 transcriptional activity was increased in an FKBP52-dependent manner, whereas it was abolished by the overexpression of the mutants. In human pregnancy, trophoblast cells invade into the uterine wall by an AP-1 mediated mechanism. Therefore, we analyzed AP-1 modulation by FKBP52 in an in vitro choriocarcinoma model (BeWo cells). ERK1/2 activation, which leads to AP-1 activation, was evaluated at protein level. FKBP52 stabilized ERK1/2 phosphorylation over time. To analyze the effects of these regulatory events, IL-6 secretion (by ELISA assay) and MMP-2 proteolytic activity (by zymography) were analyzed. Both IL-6 secretions to the medium and MMP-2 enzymatic activity were greatly increased by FKBP52 overexpression, whereas they were abrogated by the PPIase mutants. In summary, in this study we demonstrate for the first time that FKBP52 enhances ERK1/2-signalling and AP-1 transcription activity. FKBP52 effect requires binding to Hsp90 via TPR domains, and also the PPIase enzymatic activity. We conclude that FKBP52 is a novel positive regulator of AP-1.

## LONG TERM OVARIAN HORMONE DEPRIVATION INDUCES MITOCHONDRIAL BIOENERGETIC DECAY AS WELL AS CHANGES IN MITOCHONDRIAL MEMBRANE LIPID PROFILE AND DNA REPAIR MECHANISMS IN THE HIPPOCAMPUS

**SANDRA ZÁRATE**

*Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Instituto de Investigaciones Biomédicas (INBIOMED), Facultad de Medicina, Buenos Aires, Argentina.*

Mitochondrial dysfunction is a common hallmark in aging. In the female, reproductive senescence is characterized by loss of ovarian hormones, many of whose neuroprotective effects converge upon mitochondria. Mitochondria functional integrity is dependent on membrane fatty acid and phospholipid composition as well as mitochondrial DNA (mtDNA) stability, parameters also affected during aging. The aim of this work was to study the effect of long-term ovarian hormone deprivation upon mitochondrial function and its putative association with changes in mitochondrial membrane lipid profile and in mtDNA repair mechanisms the hippocampus, an area primarily affected during aging and highly responsive to ovarian hormones. To this aim, Wistar adult female rats were ovariectomized or sham-operated. Twelve weeks later, different parameters of mitochondrial function ($O_2$ uptake, ATP production, membrane potential, respiratory complex activities), membrane phospholipid content and composition as well as single steps in the main mtDNA repair pathway were evaluated in hippocampal mitochondria. Our results show that chronic ovariectomy reduced mitochondrial $O_2$ uptake and ATP production rates and induced membrane depolarization during active respiration without altering the activity of respiratory complexes. Mitochondrial membrane lipid profile from ovariectomized rats showed no changes in cholesterol levels but higher levels of unsaturated fatty acids, rendering them more prone to peroxidation. Interestingly, chronic ovariectomy also reduced cardiolipin content and altered cardiolipin fatty acid profile. mtDNA repair pathway was also altered in hippocampal mitochondria from ovariectomized rats. Our results show that chronic ovarian hormone deprivation induces mitochondrial bioenergetics dysfunction and changes in the mitochondrial membrane lipid profile and mtDNA repair mechanisms comparable to an aging phenotype. The maintenance of membrane properties and mtDNA integrity emerge as putative therapeutic targets worth exploring to avoid early impairments in mitochondrial energy expenditure that affects the high-energy demanding brain after ovarian hormone natural or surgical loss.

## (1031) EFFECT OF TANKYRASE INHIBITION IN OVARIAN CANCER CELL LINES AND NOTCH SYSTEM CROSSTALK

**SEBASTIAN BOCCHICCHIO[1], MARTA TESONE[1], GRISELDA IRUSTA[1]**

*[1]Laboratorio de Fisiología y Biología Tumoral del Ovario. Instituto de Biología y Medicina Experimental (IBYME - CONICET), Buenos Aires, Argentina*

Notch and Wnt/β-catenin are highly conserved pathways which regulate proliferation, apoptosis and differentiation. While Notch system has widely been demonstrated to be involved in ovarian cancer, Wnt/β-catenin pathway has been poorly studied in these tumors. Besides, there is little evidence that suggests a crosstalk between them. We analyzed the effect of inhibiting these two pathways and their interaction in ovarian cancer cell lines. Two human ovarian tumor cell lines, a human granulosa-like tumor cell line (KGN) and a human ovarian adenocarcinoma cell line (IGROV-1) were incubated in the presence of a Wnt inhibitor (XAV939: 1, 10, 20 and 50 μM), a Notch inhibitor (DAPT: 15, 20 μM) or both. We evaluated the involvement of Wnt/β-catenin pathway and a crosstalk with Notch system in cellular proliferation. Our results show a significant decrease in proliferation when IGROV-1 cells were incubated in the presence of XAV939 (10, 20 and 50 μM ) or DAPT (15, 20 μM). There was also a significant decrease in β-Catenin and Cyclin D1 levels together with an increase of total Axin when treated with XAV939. KGN cells also showed a significant decrease in proliferation after incubation with XAV939 (50μM). Most importantly, when IGROV-1 and KGN cells were incubated in the presence of both inhibitors, there was a synergistic decrease in proliferation suggesting a novel crosstalk between these pathways in ovarian cancer cell lines. We also tested a Wnt/β-Catenin pathway activator: LiCl. At low concentrations (10, 100 μM), KGN cells proliferation increased while β-Catenin levels remained constant. On the contrary, when KGN cells were incubated in the presence of high concentrations of LiCl (5, 10 mM), cell proliferation decreased significantly as well as total Nf-κβ, while β-Catenin levels increased. In conclusion, we demonstrate a clear involvement of Wnt/β-catenin pathway in ovarian tumor cell proliferation and suggest an interaction between this pathway and Notch system.

## CONTROL OF PIP3 LEVELS BY PI3K ALPHA IN HEALTH AND DISEASE

**IGNACIA ECHEVERRIA[a,b], EVAN BROWER[c,d], DANIELE CHAVES MOREIRA[a,e], YUNGLONG LIU[a], MICHELLE MILLER[a,f], B. VOGELSTEIN[c], S. B. GABELLI[a,g], AND L. M. AMZEL[a]**

*[a] Department of Biophysics and Biophysical Chemistry, Hopkins University School of Medicine, Baltimore, MD 21205, USA, [b] Department of Chemistry and Biochemistry, University of Maryland, College Park, Maryland 20742, USA, [c] Ludwig Center for Cancer Genetics and Therapeutics and Howard Hughes Medical Institute at the Hopkins-Kimmel Cancer Center, University School of Medicine, Baltimore, MD 21231, USA, [d] Present address: Paragon Bioservices, Baltimore, MD, USA, e Present address: Universidade Federal do Paraná, Department of Cell Biology, Brazil, [f] Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, 381 Royal Parade, Parkville, Victoria 3052, Australia, [g] Department of Medicine and Department of Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland 21287, USA.*

Phosphatidylinositide-3-kinase-a (PI3Ka) is a lipid kinase that catalyzes the phosphorylation of PIP2 to produce PIP3 in response to phosphorylated receptor tyrosine kinases (RTK) or their substrates. The increased levels of PIP3 initiate a number of signaling pathways by recruiting other kinases, such as Akt, to the plasma membrane leading to increased cell survival, cell motility, cell metabolism, and cell cycle progression. Levels of PIP3 are control by a balance between its production by PI3K and his hydrolysis back to PIP2 by the phosphatase PTEM, a tumor suppressor factor.

PI3Kα is composed of two subunits, p110 and p85, each comprising five domains. It is frequently mutated in many cancer types and the mutations increase PI3K kinase activity and provide growth advantages to the respective clones.

Several atomic resolution structures of the enzyme reveal that the enzyme has a complex architecture in which each domain interacts with several domains of the same or the other subunit. Structural and biochemical data show that physiological activation, as well as activation by some oncogenic mutations, involves relief of autoinhibition by dislodging the inhibitory nSH2 domain of the regulatory subunit p85 from its inhibitory position. Computational studies show that most of these effects involve, in addition to structural changes, modifications of the dynamics of the protein that alter the relative stabilities of the different states accessible to the enzyme.

Recent progress toward determining the mechanism of activation benefited from two developments: the determination of the structure PI3K bound to short chain phosphoinositides, and the characterization of the conformations accessible to the activation loop in molecular dynamics simulations.

**SAIC SYMOPOSIUM I**

## SOLID TUMORS
## THE PATHOGENESIS OF CASTRATE RESISTANT PROGRESSION OF PROSTATE CANCER IN BONE

**NORA M. NAVONE**

*Dept. Genitourinary Medical Oncology, MD Anderson Cancer Center, Houston, Texas USA*

Bone metastases typically develop in patients with advanced prostate cancer (PCa). These metastases are osteoblastic (bone-forming) and constitute the main cause of morbidity and mortality of PCa. Androgen deprivation is commonly used to treat bone metastases of PCa, but responses to such therapy are short, and eventually, the disease progresses to a castration-resistant form. Bone is the primary site of castration-resistant PCa progression, and clinical and laboratory investigation suggests that the PCa induced new bone formation contributes to PCa growth. Further development of therapies for bone metastases of PCa requires an understanding of the mechanisms underlying the growth of CRPC in bone.

The fibroblast growth factor (FGF)/FGF receptor (FGFR) complex, a signaling axis that typically mediates epithelial–stromal cell interactions (FGF axis), is central to prostate development, and is commonly altered during PCa progression. Recent studies by our group and others have implicated the FGF axis in the pathogenesis of PCa progression in bone, identified the FGF axis as a candidate target for therapy. Blockade of FGFRs with dovitinib (TK1258, Novartis Pharma), a receptor tyrosine kinase inhibitor (TKI) with potent activity against FGFR and vascular endothelial growth factor receptor has clinical activity in a subset of men with castration-resistant PCa and bone metastases. Therapy responses in these patients are associated with improvements in bone scan findings, lymphadenopathy, and tumor-specific symptoms without a proportional decline in PSA level. In fact, only a reduction in bone-specific alkaline phosphatase level was predictive of increased median treatment durations (23.6 weeks versus 10.6 weeks in those without a reduction in this level; P=0.056 [Wilcoxon rank sum test]). Integrated analyses of clinical and preclinical studies suggest that FGF signaling mediates a positive feedback loop between PCa cells and bone cells and that blockade of FGFR1 in osteoblasts partially mediates the antitumor activity of dovitinib by improving bone quality and by blocking PCa cell–bone cell interaction

Similar therapeutic activity without proportional reduction in PSA blood levels was observed with Alpharadin (Radium-223 dichloride), a bone seeking alpha emitter (FDA-Approved for castration-resistant PCa with bone metastases). These clinical findings support our hypothesis that a specific interaction between PCa and bone cells favors PCa growth in bone. Understanding the mechanism underlying the interaction between PCa cells and bone cells will help identify markers of progression and more effective targets for therapy.

## GENOMICS-BASED IDENTIFICATION OF CLINICALLY-INFORMATIVE LUNG CANCER BIOMARKERS

**ANA I. ROBLES**

*Laboratory of Human Carcinogenesis, National Cancer Institute, NIH, Bethesda, Maryland, USA*

Lung cancer is the leading cause of cancer-associated deaths worldwide, despite a slow but continuous decline in incidence and mortality in Western countries over the past two decades. Global variations in lung cancer incidence largely follow historical patterns of smoking, and incidence and mortality rates are still on the rise in Asia and some countries in Latin America and Africa, where the smoking epidemic began later. Most lung cancer patients are diagnosed with locally advanced or metastatic disease, with few therapeutic options and a dismal survival rate. Cigarette smoking is the major risk factor for lung cancer and other smoking-related diseases. Even as this risk gradually decreases after smoking cessation, former smokers account for most new lung cancer diagnoses. Thus, lung cancer screening efforts have focused on older individuals with a history of heavy smoking. Implementation of lung cancer screening using Low-Dose Computed Tomography (LDCT) is expected to result in a greater proportion of lung cancers being diagnosed at an early, operable, stage. Still, the overall rate of recurrence for surgically treated Stage I lung cancer patients is up to 30% within 5 years of diagnosis. Clinically validated biomarkers for lung cancer detection, and prediction of patient prognosis and response to therapy may help patient management.

Comprehensive genomic characterization has advanced our understanding of the complex changes as-

sociated with cancer development. For lung cancer, these data have revealed vast genetic heterogeneity that poses at the same time a challenge and an opportunity to selectively target specific molecular alterations and disease subtypes. The development of molecularly targeted therapies against oncogenes that are somatically activated or translocated in tumors has revolutionized the treatment paradigm for lung cancer patients. Clinical management is increasingly based on molecular parameters in addition to histological classification, despite the fact that over 50% of lung adenocarcinomas show no clinically actionable DNA alterations. In addition, recent promising results of T cell-based immunotherapy associated high mutational burden in lung cancer patients suggest that exome-guided neoantigen identification may improve treatment responses. Thus, the implementation of targeted therapies and immunotherapy rely on accurate stratification of patients based on molecular data.

Biomarkers of lung cancer detection, patient prognosis and response to therapy have been identified through bioinformatics and statistical analyses of exome sequencing, DNA methylation, gene and miRNA expression data. Beyond a more comprehensive understanding of the molecular taxonomy of lung cancer, these biomarkers can have clinical utility for the management of early stage lung cancer patients. First, tumor-derived circulating biomarkers associated with lung cancer risk could help prioritize individuals for LDCT screening. Second, biomarkers could aid in detection of lung cancer or help discriminate malignant nodules from benign or indolent lesions. Third, biomarkers that molecularly categorize Stage I patients after tumor resection could help identify high-risk patients who may benefit from adjuvant chemotherapy or innovative immunotherapy.

## SAIC SYMOPOSIUM II

## ENDOCRINOLOGY, *IN MEMORIAM* DR. CARLOS LANTOS

### ROLE OF MICRORNAS IN CARDIAC INJURY AND DYSFUNCTION IN PRIMARY ALDOSTERONISM

**DAMIAN G. ROMERO**

*Department of Biochemistry, Cardio-Renal Research Center, and Women's Health Research Center, University of Mississippi Medical Center, Jackson, MS, USA*

Primary aldosteronism is characterized by excess autonomous secretion of aldosterone (ALDO) independent of the renin-angiotensin system and accounts for ~10% of hypertensive patients. Excess ALDO, inappropriate for the salt intake status, causes hypertension and cardiac hypertrophy, inflammation and fibrosis that lead to cardiac dysfunction. The molecular mechanisms that trigger the onset and progression of ALDO-mediated cardiac injury and dysfunction are poorly understood.

MicroRNAs (miRNAs) are endogenous, small, non-coding RNAs that have important roles in development and cell proliferation and differentiation. miRNAs downregulate the expression levels of specific proteins by translational repression or mRNA degradation. Several miRNAs have been implicated in diverse cardiac pathologies in humans and animal experimental models, yet very little is known about their regulation and role in ALDO-mediated cardiac injury and dysfunction.

To analyze the regulation of miRNAs in ALDO-mediated cardiac injury, we performed a time-series analysis of left ventricle (LV) miRNA expression. Eight-week old uninephrectomized male Sprague Dawley rats were treated with ALDO (0.75 µg/h) infusion and SALT (1.0% NaCl/0.3% KCl) in the drinking water for up to 8 weeks. miRNA expression was analyzed by miRNA microarrays followed up by qRT-PCR and Northern-blot validation. ALDO/SALT time-dependently modulated the expression of 96 miRNAs in the LV. microRNA-21 (miR-21) was the most upregulated miRNA after 2 weeks of treatment and remained elevated until the end of the study. LV ALDO/SALT-mediated miR-21 upregulation was specific to the LV cardiac chamber and prevented by co-treatment with a triple antihypertensive therapy suggesting that such increase is blood pressure-dependent.

To elucidate the role of miR-21 in ALDO/SALT-mediated cardiac injury, miR-21 was downregulated using chemically modified antisense oligonucleotides (antagomirs) in ALDO/SALT-treated rats. miR-21 downregulation exacerbated ALDO/SALT-mediated cardiac and LV hypertrophy and cardiac dimensions determined by echocardiography. Furthermore, miR-21 downregulation increase cardiac fibrosis markers (collagen I AND III) gene expression, interstitial and perivascular fibrosis, and OH-proline content. On the other hand, miR-21 downregulation attenuated ALDO/SALT-mediated cardiac inflammation markers (Tgfb2, IL-1$\beta$, MCP-1) gene expression. miR-21 downregulation exacerbated ALDO/SALT-mediated cardiac dysfunction as observed by a further decrease in cardiac output and fractional shortening.

In summary, these results suggest that ALDO/SALT-mediated cardiac miR-21 upregulation may be a compensatory mechanism that mitigates ALDO/SALT-mediated cardiac deleterious effects. We speculate that miR-21 supplementation would have beneficial effects in reverting or mitigating cardiac injury and dysfunction in patients with primary aldosteronism.

### IMPACT OF THE ENDOCRINE SYSTEM ON AGEING

**JESÚS TRESGUERRES**

*Dept of Physiology Medical School, University Complutense of Madrid, Spain*

The ageing process is apparently due to the accumulation of oxidative damage in cells and molecules that in turn are leading to vascular alterations, infections and degenerative alterations of the Central Nervous System (CNS). Skin has been shown to be also affected .The age related reduction in the activity of the immune system is linked with an enhanced susceptibility to infections, autoimmune disorders and cancer Aging is also associated with the reduction in the secretion of several hormones including GH, melatonin and estrogens, and some of the aging associated alterations could be at least partially due to this fact.

Growth hormone (GH) exerts effects on the CNS, the immune system, the skin and on the vascular endothelium. In addition, the fall in estrogens induced by ovariectomy in females or menopause in women is able to induce further deleterious effects on different organs and systems. Melatonin shows also effects on all the above mentioned organs and systems and shows also a reduction with age. The aim of our study has been to investigate the effect of chronic replacement therapy with physiological doses of GH, melatonin, estrogens and phytoestrogens on vascular function and structure, bone physiology, the immune system and on some parameters related to oxidative stress and inflammation in the CNS and liver using both a rat model of aging and a mouse model of accelerated senescence.

An increase in the aortic media thickness was seen in old animals, which showed also a reduction in the vasodilatory response to isoprenaline ($p<0.001$) as compared to young animals. GH treatment partially restored both. A reduction in the total number of neurones in old animals of both genders ($p<0.005$) has been detected together with a marked reduction of neurogenesis. Treatment with GH increased the total number of neurones without affecting neurogenesis whereas melatonin administration was able to enhance neurogenesis without affecting total number of neurones in the same way as estrogens and phytoestrogens. Markers of oxidative stress, inflammation and apoptosis have been determined in the CNS and also in the liver, showing a marked increase with age. Measurements of TNF alpha, several interleukins, iNOS, eNOS, NFkB,and parameters of apoptosis such as BCL2, BAX, BAD and others, were performed by ELISA , but also using molecular biology (PCR and Western blot) GH, melatonin and estrogen treatments were able to reduce all pro inflammatory, pro oxidative stress and pro apoptotic substances and to stimulate anti-inflammatory and anti apoptotic markers. All these actions lead in the liver to an increase in ATP formation. Age linked skin alterations, such as increased fat dermis content and reduced epidermal thickness showed a marked improvement after GH, melatonin and estrogen treatments. Keratinocytes in culture obtained from old animals presented enhanced oxidative stress and apoptosis and reduced BCl2 that were restored with GH, melatonin and estrogens.

In conclusion, chronic GH replacement to old experimental animals showed beneficial effects on body composition, vascular function and morphology, CNS and immune parameters**.** Melatonin has also shown beneficial effects on the skin, immune parameters and bone. Estrogens and isoflavones given to ovariectomized females were able to prevent oxidative changes in liver tissue and hepatocytes and also in keratinocytes in culture. All these results can be used in some of the age associated alterations, also in the human, as will be discussed during the presentation.

## SAIC SYMPOSIUM III

## NEURODEGENERATION AND NEUROPROTECTION

### ROLE OF ASTROCYTES IN BRAIN ISCHEMIA: ACTIVE PLAYERS OR MERELY BYSTANDERS OF THE INNATE IMMUNE SYSTEM?

**ALBERTO JAVIER RAMOS**

*Laboratorio de Neuropatología Molecular, IBCN UBA-CONICET, Facultad de Medicina, Universidad de Buenos Aires.*

For almost a century astrocytes were disregarded when studying the Central Nervous System (CNS) neuronal complexity. The prevalent vision during that time simply considered astrocytes to be the brain *glue*, and

their function to give metabolic and physical support to neurons. It was not until the last decade that the glia field, and specifically the study of astroglial functions and heterogeneity, has been further explored using state-of-the-art tools. Various astroglial roles have been uncovered; including their ability to modulate neuronal physiology, synaptic transmission, neurogenesis, microcirculation, CNS development, and interaction with the innate immune system. Several of these recently described astroglial roles are very important; not only are they essential for the development and function of the healthy CNS, but they are also deeply involved in the processes occurring in the injured or diseased CNS.

Since the CNS is believed to have its own professional immune cells –microglia–, with only a limited participation of peripheral blood-derived immune cells, astroglial direct participation in the innate immunity response is still today under controversy. Innate immunity activation in the absence of infection relies on the Damage Associated Molecular Patterns (DAMP) release, which behave as ligands of the Pattern Recognition Receptors (PRR), such as Toll-like (TLR), RAGE and others. During the last years our work has been focused in studying the astroglial participation in the innate immunity response after focal brain ischemia. We have shown that astrocytes essentially behave as facultative cells of the innate immunity response that classically follows brain ischemia. Following experimental brain ischemia or *in vitro* OGD, astrocytes upregulate the expression of the PRR, including TLR2, TLR4 and RAGE. This effect parallels the well known reactive gliosis, a phenomenon largely observed by pathologists in the injured brain. Using gain- and loss-of-function studies achieved with plasmid transfection and knockout mice, we have shown that TLR expression in astrocytes facilitates astroglial response to PAMP and DAMP. TLR4 overexpression and activation by ligand also produces a sustained NF-$\kappa$B activation with increased expression of the proinflammatory cytokines IL-1$\beta$ and TNF$\alpha$, indicative of a proinflammatory polarization of reactive astrocytes. Furthermore, we have recently shown that Triggering Receptor Expressed on Myeloid cells (TREM-2) is also expressed in astrocytes after OGD, and that TREM-2 activation by antibody crosslinking limits LPS-induced NF-$\kappa$B activation. This is another typical response of cells committed to the innate immune response.

Taken together, our results show that brain ischemia induces reactive gliosis, which involves the expression of a large number of genes. These include the PRR, a family of receptors that enable astroglial direct participation in the innate immunity response, by sensing the DAMP released by the necrotic ischemic core. In this scenario, astrocytes and microglia act both as sensors but also as effectors of the innate immunity response following ischemia. Having in mind the important role of brain inflammation in the injured brain, the DAMP/PRR/NF-$\kappa$B pathway emerges as a tempting target to develop new treatment strategies.

## PROTECTIVE ROLE OF SEX STEROIDS AND DERIVATIVES IN MOTONEURON DEGENERATION: A TRANSLATIONAL PERSPECTIVE

**MARIA CLAUDIA GONZÁLEZ DENISELLE**

*Instituto de Biología y Medicina Experimental (IBYME), CONICET y Departamento de Ciencias Fisiológicas, Facultad de Medicina, Universidad de Buenos Aires (UBA)*

Amyotrophic lateral sclerosis (ALS) is a fatal degenerative disorder with onset in adulthood, characterized by the selective and progressive death of upper and lower motoneurons, leading to progressive paralysis of voluntary muscles. Respiratory impairment is a major cause of morbidity and mortality in these patients and respiratory function is directly related to survival time. Epidemiological studies have shown male predominance in ALS, suggesting the participation of hormonal factors in disease development. Changes of the hypothalamic-pituitary-adrenal (HPA) axis have been observed in ALS patients (Gargiulo Monachelli et, 2011; Spataro et al, 2015). The Wobbler mouse has successfully been used as an animal model for ALS in the investigation of both pathology and therapeutic treatment. Previous limited studies have also reported steroidal hormone deregulation in Wobblers. Clinically, Wobblers develop forelimb muscle atrophy and gait disturbances. In common with other rodent models and ALS, Wobbler present abnormalities of TDP43 (transactive response DNA binding protein) and ubiquitination, upregulation of the tumor necrosis factor alpha and cortical hyperexcitability (Bigini et al. 2008; Dennis and Citron 2009). Sex steroids such as progesterone have neuroprotective effects and others such as glucocorticoids at sustained high levels have neurotoxic effects. Progesterone therapy provides beneficial effects in Wobbler and superoxide dismutase 1 (SOD1) transgenic mice, reducing molecular and functional abnormalities of motoneurons (Gonzalez Deniselle et al, 2012; Kim et al, 2013).

Since progesterone is metabolized in the central nervous system (CNS) into 5 alpha-dihydroprogesterone (DHP) and 3a, 5a tetrahydroprogesterone or allopregnanolone (ALLO), the effects of this steroid may be partly mediated by its reduced derivatives. In this regard, ALLO is also neuroprotective in nerve regeneration and myelination, excitotoxic damage, ischemic stroke and disorders such as Alzheimer disease (Guennoun et al. 2015; Irwin et al 2014). We studied if ALLO, a reduced metabolite endowed with gabaergic activity, also prevents neuropa-

thology in Wobbler mice after an acute or chronic therapy. Untreated Wobblers showed increased serum levels of progesterone and its reduced derivatives DHP and ALLO vs. control animals. Treatment with ALLO elevated its levels in serum without changing the concentration of progesterone and DHP. Parameters measured in the spinal cord included brain-derived neurotrophic factor (BDNF) mRNA, p75 neurotrophin receptor (p75NTR) and TrkB receptors, the phosphorylation of the downstream AKT and the stress activated kinase JNK. Untreated Wobblers showed reduction of BDNF, TrkB, and pAKT and high expression of pJNK and p75NTR. With the exception of BDNF, these alterations were prevented by an acute ALLO treatment. On the other hand, chronic administration of ALLO enhanced BDNF mRNA and attenuated pJNK and muscle weakness, Thus, ALLO decreased motoneuron pathology and delayed disease progression. Downregulation of p75NTR may provide adequate neuroprotection at early stages of the disease. Since long-term steroid treatment also increased BDNF mRNA and reduced pJNK, both ALLO-treatment protocols should be combined in order to provide neuroprotection in motoneuron disease. In the animal model, our data shows that ALLO, a $GABA_A$ receptor agonist, might directly delay the progression of the Wobbler disease, without being metabolized into progesterone receptor agonists, DHP and progesterone.

Changes in serum concentration of sex steroid hormones and glucocorticoids also occur in ALS patients. Increased serum cortisol levels have been found in these patients, suggesting HPA axis dysfunction. Regarding sex steroids, patients with a slow and intermediate disease course showed higher progesterone serum levels than those with a rapid disease course. On the other hand, the finding of a low index-to-ring finger length ratio (2D:4D ratio) in ALS suggested an increased prenatal exposure to androgens, which might play a role in motoneuron vulnerability in adulthood. In this regard, we searched for the relationship between the circulating levels of gonadal and adrenal steroids and respiratory function, considering that respiratory failure is one frequent cause of death in ALS. Testosterone serum levels declined with age in controls but not in ALS patients. Higher dehydroepiandrosterone sulfate (DHEAS)/cortisol ratio showed a positive correlation with respiratory function, whereas elevation of circulating testosterone, as well as low progesterone/free testosterone ratio were associated with a rapid worsening of the respiratory function (Gargiulo Monachelli et al, 2014). In ALS patients, circulating gonadal and adrenal steroids are differentially expressed relative to controls, which might influence respiratory function and outcome. DHEAS or progesterone may provide a protective function, while other steroids like testosterone or cortisol probably have a negative influence.

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## (1004) PROGESTERONE HAS A NEUROPROTECTIVE EFFECT AND PREVENTS DEPRESSION SIGNS IN A MALE RAT DEPRESSION LIKE MODEL

**VANINA ANABEL VILLEGAS, MARÍA BELÉN MULLE BERNEDO, SEBASTIÁN GARCÍA, ANTONELLA ROSARIO RAMONA CÁCERES GIMENEZ, ROBERTO YUNES, RICARDO CABRERA**

*Instituto de Investigaciones Biomédicas - Facultad de Ciencias de la Salud- IMBECU-CONICET*

Depression is one of the psychiatric disorders with the highest incidence in the recent decades. It has been recently related to different neurodegenerative diseases such as Parkinson. These pathologies often appear associated to a premotor sign such as depression and decreased cognitive performance.

The objective was to study and evaluate if the treatment with the neuroactive steroid progesterone could prevent the development of premotor early signs of neurodegenerative diseases in a model of catecholaminergic depletion by reserpine. Sprague-Dawley male rats (250-350 g), between 60 and 90 days old were used. The experimental groups were: C (saline), R (reserpine 0.1 mg/kg/sc, 10 injection over the course of 20 days), P (progesterone 4 mg/kg/sc) alone, 5 days after de experiments and PP + R (reserpine 5 days after a previous dose of progesterone). During the course of reserpine treatment the animals were evaluated in the catalepsy test. Forced swimming and novel object recognition were tested before the appearance of the motor signs. Data were analysed by ANOVA-1 and Tukey`s post hoc.

A significant decrease ($p<0.05$) was observed between the R vs C groups in all evaluated behavioural parameters. There were no significant differences in the catalepsy time in the PP+R vs C. Although PP+R vs R, showed a significant increase in the time spend swimming on the

force swimming test ($p<0.05$) and in the discrimination index ($p <0.05$) in novel object recognition.

We conclude that a previous progesterone treatment can avoid depression like behaviour and improves short time memory and the on/ off effect on locomotor activity. Progesterone exerts a neuroprotective effect against the reserpine treatment, preventing cognitive and depression premotor disorders induced by catecholamine depletion.

## STRUCTURAL COMPOSITION AND FUNCTIONAL CHARACTERISTICS OF THE GUT MICROBIOME IN AUTOIMMUNE DISEASES

**SERGIO E. BARANZINI**

*Department of Neurology, University of California San Francisco*

A major role of the human gut microbiota is to regulate both innate and adaptive immune responses during health and disease. While most studies of the human microbiome to date have focused on analyzing microbial population structures, it is equally important to investigate how variation in microbial abundance and composition affects host functions. Responses by primary human immune cells to gut microbiota is one model for studying microbial immunoregulation. Growing evidence of microbiome alterations in multiple human autoimmune diseases and specifically of microbial regulation of immune responses in experimental autoimmune encephalomyelitis (EAE) led us to investigate changes in intestinal microbiota as a potential pathogenenic mechanism in multiple sclerosis (MS), an autoimmune disorder of the central nervous system.

MS-like symptoms in EAE can be exacerbated by T helper 1 and 17 (Th1 and Th17) responses, and modulated by Tregs. Recent studies compared the microbiota of MS patients to healthy controls, and while these studies were performed with small sample sizes and did not discriminate for treatment with disease modifying drugs, a consistent pattern of modest dysbiosis emerged.

We performed microbiome analysis by 16S rRNA gene sequencing of stool samples from 68 untreated MS patients and 64 healthy controls. Although no major shifts in community structure were found when compared to healthy controls, we identified specific microbial taxa significantly associated with MS. We found that *Akkermansia muciniphila* and *Acinetobacter calcoaceticus*, which are increased in MS, induce a proinflammatory response from human PBMCs. In contrast, *Parabacteroides distasonis*, which is reduced in MS, promotes regulatory T cell (Tregs) differentiation.

In addition, physical fractionation of bacterial communities resulted in the selection of certain populations whose abundance clearly discriminate samples of cases and controls.

Finally, microbiota transplants from MS patients into germ-free mice results in more severe experimental autoimmune encephalomyelitis and reduced Tregs compared to controls. This study identifies specific human gut bacteria that regulate adaptive autoimmune responses, suggesting therapeutic targeting of the microbiota as a novel treatment for MS.

# SAIC-SAFE SYMPOSIUM (IV)

# CARDIAC FUNCTION AND REGENERATION

## ROLE OF P38MAPK PATHWAY IN CARDIAC POSTNATAL DEVELOPMENT

**GUADALUPE SABIO**

*CNIC, Instituto de Salud Carlos III, Madrid, Spain*

Cardiac growth is tightly regulated to ensure that the heart reaches its appropriate size. Cardiomyocytes rapidly proliferate during fetal life, but soon after birth differentiated cardiomyocytes enter a postmitotic state and the ability to proliferate is lost. Postnatal cardiac growth is therefore mainly achieved through increases in cell size (physiological hypertrophy) associated with increased protein synthesis together with the expansion of non-myocyte populations. Hypertrophic growth is also the adaptive response of cardiomyocytes to stress stimuli, including cardiac pressure or volume overload, cytoskeletal abnormalities, and intrinsic contractility defects.

Disrupted organ growth underlies the development of several diseases. Hypertrophy underlies postnatal heart growth and is activated after stress. Here we will discuss the implication of p38 pathway in this process. The relative involved of the upstream kinases MKKs in this process.

## MESENCHYMAL AND CARDIAC DIFFERENTIATION OF PLURIPOTENT STEM CELLS: OVERCOMING HURDLES TO CLINICAL APPLICATION

**SANTIAGO MIRIUKA**

*FLENI Foundation and CONICET, Buenos Aires, Argentina*

Pluripotent stem cells are anchored at an early developmental stage. Once signals that keep them in an undifferentiated stage are withdrawn, they rapidly differentiate into any cell of the three germinal layers. This property is widely used for research in potential regenerative therapies, understanding the early differentiation processes in human development, and to in vitro modeling disease. The discovery ten year ago of cell reprogramming has foster many laboratories to work with pluripotent stem cells.

Our lab has been interested in mesoderm differentiation, particularly in cardiomyocyte development starting from pluripotent stem cells. Our latest research is focused in the microRNA regulation of mesoderm and cardiac differentiation. By using several bioinformatic tools, we have build a comprehensive mirnome of the regulating microRNA in this landscape. This mirnome shows that several clusters and family microRNA forms a complex network for mRNA expression. Examining the mirnome, it is possible to identify different behaviors in the expression of the individual microRNA. Interesting, families and clusters of microRNA behave in a similar way. Gene ontology analysis of these families and clusters reveals subset of microRNA that specifically regulates major signaling pathways of pluripotent stem cell and mesoderm and cardiac differentiation.

The mirnome is, however, a part of the complex regulation of mesoderm and cardiac specification. We face now two challenges. First, to analyze microRNA families as a whole, and not individual microRNAs. Second, to apply a broader view in order to understand the regulation of mesoderm and cardiac differentiation. We are therefore on the way to analyze the lncRNA population and to reveal how it relates to the mirnome.

## (218) DIFFERENCES IN THE PROTECTION MECHANISMS OF PRECONDITIONING AND POSTCONDITIONING INDUCED BY VAGAL STIMULATION IN MYOCARDIAL INFARCTION IN MICE.

**JAZMÍN KELLY[1], BRUNO BUCHHOLZ[1], MARINA MUÑOZ[2], EDUARDO BERNATENÉ[1], NAHUEL MÉNDEZ DIODATI[1], DANIEL GONZÁLEZ MAGLIO[3], FERNANDO P. DOMINICI[2], RICARDO J. GELPI[1]**

*[1]Institute of Cardiovascular Physiopathology, Faculty of Medicine, University of Buenos Aires. [2]Institute of Chemistry and Biological Physicochemistry (IQUIFIB), Faculty of Pharmacy and Biochemistry University of Buenos Aires. [3] Department of Immunology, Institute of Studies of Humoral Immunity (IDEHU UBA-CONICET), Faculty of Pharmacy and Biochemistry, University of Buenos Aires.*

We have previously proven that vagal stimulation (VS) decreases infarct size both when applied before ischemia and during reperfusion. However, their mechanisms are yet unknown. Thus, our objective was to study the molecular pathways involved in the protection of preischemic VS (pVS) and reperfusion VS (rVS). Mice were randomly assigned to the following groups: Sham (n=6); 30 min of regional myocardial ischemia and 15 min of reperfusion without VS (I/R, n=6); with 10 min of pVS with (n=6) and without (n=6) muscarinic blockade with atropine; 10 min of rVS with (n=6) and without (n=6) alpha-7 nicotinic blockade with MLA. Left ventricle samples were taken for Western blotting at the end of reperfusion. IL-6 levels were assessed by ELISA in myocardium and plasma after 2 h of reperfusion. pVS increased Akt phosphorylation in comparison to the I/R and Sham group (4.2±0.64; 2.15±0.17 and 0.80±0.13 respectively). This was reversed with administration of atropine (1.13±0.34). GSK-3β had a similar increase (4.65±0.52; 2.91±0.36 and 1.05±0.05; respectively) and reversed as well with atropine (1.44±0.33). There were no significant differences in ERK1/2, JAK2 and STAT3 phosphorylation. rVS produced a non significant rise in JAK2 phosphorylation compared to I/R (2.36±0.40 and 1.64±0.2; respectively). There were no significant differences between I/R, rVS and rVS+MLA groups in Akt, GSK-3β, ERK1/2 and STAT3 phosphorylation. Additionally, rVS did not reverse the increase in IL-6 produced during ischemia/reperfusion. In conclusion, pVS reduces infarct size in mice by muscarinic activation of the Akt/GSK-3β pathway. Conversely, rVS protection seems to be mediated by alpha-7 nicotinic activation of the JAK2 pathway, independently of local myocardial and systemic anti-inflammatory responses.

## REGULATION OF THE CARDIAC SODIUM CHANNEL NAV1.5 BY CALMODULIN AND CALCIUM

**JESSE YODER[A], GORDON TOMASELLI[B], SANDRA GABELLI[A,B] AND L. MARIO AMZEL[A]**
*Departments of Biophysics and Biophysical Chemistry[a] and Medicine[b]*
*Johns Hopkins University School of Medicine, Baltimore, MD, USA*

Voltage gated sodium channels (Nav) are membrane bound proteins that allow rapid $Na^+$ influx during the leading edge of action potentials in neural, cardiac, and muscle cells. Activation of the channels in response to membrane depolarization leads to the opening of their central pore allowing $Na^+$ influx, followed by a fast inactivation mechanism that occludes the still-open pore, ablating ion flow. The biological regulation of the channels is complex. It includes transitions from the resting-state to inactivation states such as steady-state inactivation, and the state resulting from $Ca^{2+}$-dependent inactivation (CDI) and FHF (a co-protein) dependent inactivation.

The molecular mechanisms of activation and inactivation are thought to involve in part the Nav cytosolic C-terminal tail (CT-Nav) and calmodulin (CaM). CT-Nav, the cytsolic portion of the channel that follows the final trans-membrane helix, contains an EF-hand like domain (EFL) with no apparent $Ca^{2+}$-binding ability, followed by a long helix that contains an IQ-motif. Calmodulin (CaM) binds to the IQ-motif in the presence and absence of $Ca^{2+}$. $Ca^{2+}$-free CaM enhances activation of both Nav1.4 (the skeletal muscle isoform) and Nav1.5 (the cardiac isoform). Addition of calcium and the formation of $Ca^{2+}$-CaM, however, inactivates Nav1.4 but not Nav1.5.

The three dimensional structure of CT-Nav1.5 in complex with apo-CaM provides insights into possible mechanisms regulating the activation of the cardiac channels. Thermodynamic data obtained using Isothermal Titration Calorimetry (ITC) provide clues about the different responses to $Ca^{2+}$ (CDIs) of the cardiac (Nav1.5) and the muscle (Nav1.4) channels.

# SAIC SYMPOSIUM V

# EPIGENETICS

## EARLY LIFE ADVERSITIES AND THE EPIGENETIC PROGRAMMING OF GENE EXPRESSION

**EDUARDO CÁNEPA**
*Department of Biological Chemistry, School of Exact and Natural Sciences,*
*University of Buenos Aires, Argentina*

The quality of brain architecture is established early in life through a series of dynamic interactions in which environmental conditions and personal experiences have a significant impact on the establishment of genetic programming. Brain architecture is constructed over a succession of ''sensitive periods'', each of which is associated with the formation of specific circuits that underlie specific abilities. The prenatal period and the first few years of life are particularly important because vital development occurs in all domains. The brain develops rapidly through neurogenesis, axonal and dendritic growth, synaptogenesis, cell death, synaptic pruning, myelination, and gliogenesis. These ontogenetic events happen at different times and build on each other, such that small perturbations in these processes can have long-term effects on the brain's structural and functional capacity. Several longitudinal studies suggest that adverse childhood experiences are associated with changes in biological systems responsible for maintaining physiological stability through environmental changes. Children exposed to maltreatment showed smaller volume of the prefrontal cortex, greater activation of the HPA axis, and elevation in inflammation levels compared to non-maltreated children. Animal research shows that environmental toxins, stress, and poor stimulation and social interaction can affect brain structure and function, and have lasting cognitive and emotional effects. In humans and animals, variations in the quality of maternal care can produce lasting changes in stress reactivity, anxiety, and memory function in the off spring. These findings raise the intriguing question of how these experiences become incorporated at the cellular and molecular level in the brain architecture leading to long-term alterations in various functions ultimately culminating in an increased risk to mental disease. Current work suggests that epigenetic mechanisms of gene regulation could explain how early life experiences can leave indelible chemical marks on the brain and influence both physical and mental health later in life even when the initial trigger is long gone. Epigenetic regulation of gene expression therefore allows theintegration of intrinsic and environmental signals in the genome, thus facilitating the adaptation of an organism to changing environment through alterations in gene activity. In this way, epigenetics could be thought of as conferring addi-

tional plasticity to the hard-coded genome. In the context of the early life environment, epigenetic changes offer a plausible mechanism by which early experiences could be integrated into the genome to program adult hormonal and behavioral responses.

Maternal malnutrition, due to its widespread incidence, remains one of the major early life adversities affecting the development of newborn's brain. An increasing number of studies point out that the effects of early-life nutritional inadequacy are persistent and lead to permanent deficits in learning and behavior. While there is no doubt that maternal malnutrition is a principal cause of perturbed development of the fetal brain and that all nutrients have certain influence on brain maturation, proteins appear to be one of the most critical for the development of neurological functions. Studies carried out by our group in recent years demonstrated that mice subjected to perinatal protein malnutrition show a delay in their physical and neurological development and present deficiencies in their learning and memory capacities and display abnormal emotional behavior, such as anxious and depressive-like disorders. Likewise, we demonstrated that these cognitive deficiencies and behavioral modifications persist during later stages of life. Currently, we investigate the molecular bases of these cognitive and behavioral deficiencies with special emphasis on epigenetic mechanisms. We focus on the hippocampus because as part of the limbic system has a major role in cognition and mood regulation. The mice that were subjected to protein malnutrition during pre- and postnatal development have a diminished expression of immediate early genes and calcineurin when confronted with an acute stress in the adulthood and display an altered miRNA-expression profile. Additionally, these mice exhibit a fewer number of neurons in the hippocampus, especially in CA3 and dentate gyrus, regions that are critical for stress response. The deep and persistent consequences of malnutrition on the intellectual and social skills of individuals affected throughout their lives represent a huge human and economic cost. Research in this area could provide knowledge for the design of suitable social or pharmacological interventions that reverse deleterious epigenetic programming triggered by adverse conditions during early life.

## TOWARDS A BETTER UNDERSTANDING OF THE EPIGENETIC MECHANISMS UNDERLYING INTELLECTUAL DISABILITY: FUNCTIONAL CHARACTERIZATION OF THE HISTONE DEMETHYLASE PHF8

**MARIAN MARTÍNEZ-BALBÁS**

*Instituto de Biología Molecular de Barcelona (IBMB), Spanish Research Council (CSIC), Barcelona Science Park (PCB), Barcelona 08028, Spain.*

Histone methylation is a regulatory mark that serves to control the transcriptional programs. In the last years several histone demethylases (HDM) have been identified as important players in neural development and function and their molecular mechanisms of action are starting to be underscored. PHF8 is a recently identified HDM that removes H4K20me and H3K9me2 marks. Interestingly, mutations on the PHF8 catalytic domain lead to mental retardation and autism. Although the activity of PHF8 is well characterized in vitro, the molecular mechanisms responsible for its role in nervous system development and function are not clearly established. In this talk we will discuss a new function of PHF8 fine tuning the transcriptional activity of genes to properly respond to external signals.

## EPIGENETIC REGULATION IN THE HEMATOPOIETIC SYSTEM

**MARIA E. FIGUEROA**

*University of Miami Miller School of Medicine, Florida, USA*

Maintenance of the hematopoietic stem cell (HSC) pool is crucial for the production of mature blood and bone marrow cells. With age, there is loss of HSC function, exemplified by a decreased homing ability and an increased predisposition to differentiate into myeloid rather than lymphoid cells. This age-associated loss of HSC function contributes to an impaired hematopoietic system; elderly individuals have increased rates of anemia, loss of adaptive immunity, and an increased risk to develop myeloid malignancies. One such disorder is Myelodysplastic Syndromes (MDS), a heterogeneous group of malignancies seem most frequently in elderly individuals. Mutations in proteins involved in alternative splicing and epigenetic modifiers such as DNMT3A and TET2, which are frequently observed in MDS, can also occur in otherwise healthy elderly individuals. However, little is known about epigenetic deregulation in the human hematopoietic system with aging, and whether such deregulation predisposes for MDS. In order to investigate this, we examined epigenetic profiles in FACS purified HSC/HSPCs isolated from bone marrow from young (18-30 yo) and elderly (65-75 yo) healthy donors. For each age group

we performed 5-6 biological replicates of genome-wide chromatin immunoprecipitation followed by sequencing (ChIP-seq) for promoter-associated chromatin marks (HeK4me3 and H3K27me3), enhancer-associated histone modifications (H3K4me1 and H3K27ac), as well as genome-wide cytosine modifications (5-methylcytosine and 5-hydroxymethylcytosine) and gene expression profiled by next-generation RNA sequencing. We have found marked epigenetic differences during normal aging that target enhancer and promoter regions of genes involved in key pathways in development and disease. These differences consist primarily of a loss of activation marks. A subset of bivalent promoters targeting the WNT, cadherin and hedgehog signaling pathways display loss of bivalent potential with aging. Analysis of cytosine modification profiles revealed only moderate changes in 5-methylcytosine while 5 hydroxymethylcytosine was markedly increased with aging. Finally, RNA-seq analysis revealed downregulation of gene transcription and aberrant patterns of alternative splicing with aging, which targeted important hematopoietic transcription factors and epigenetic modifiers. Our findings demonstrate that even in the absence of disease, aged HSCs shows massive epigenetic reprogramming targeting important pathways in development and hematopoiesis.

## SAIC-SAFE SYMPOSIUM VI

## MOLECULAR PARASITOLOGY

### *TOXOPLASMA* HISTONE VARIANTS AND THEIR ROLE IN DIFFERENT PARASITE PROCESSES

**SERGIO O. ANGEL, LAURA VANAGAS, SILVINA S. BOGADO**

*IIB-INTECH, CONICET/UNSAM, Argentina*

*Toxoplasma gondii* is a coccidian protozoan parasite that belongs to the phylum Apicomplexa. It is estimated that toxoplasmosis exists as a chronic asymptomatic form in 5 hundred million to 1 billion of the world human population. Although infection with *T. gondii* is usually asymptomatic in most individuals, it is of great medical significance for pregnant women and immunocompromised patients. In humans, *T. gondii* infection is characterized by two stages, the rapidly growing tachyzoites, and the latent bradyzoite tissue cysts. Tachyzoites are responsible for acute illness and congenital birth defects. *T. gondii* tachyzoites contain basal levels of γH2A.X, a marker of double strand break (DSB) damage, even in normal conditions lacking a DNA damaging stress. This parallels what is seen due to fork collapse under replication-associated DNA stress in cancer cells. Bradyzoites form cysts that remain latent for many years but are still capable of converting into the destructive tachyzoite form if host immunity decreases. These two developmental stages are essential for cause and propagation of disease. Tachyzoite to bradyzoite conversion, and vice-versa, includes a high number of gene expression modifications. It is believed that the epigenetic control of gene regulation is crucial for parasite development, a process that relies on the post-translational modification (PTM) of histones and histone variant exchange. *T. gondii* possess the four canonical histones H2A, H2B, H3 and H4 and variant histones of H3 and H2A families. Concerning the H2A family, the parasite has H2AZ and H2A.X variants, a feature that is not shared by other apicomplexan parasites in which H2AX is not present. In higher eukaryotes, the role of H2A.Z is associated to transcriptional regulation, genome stability, and blocking the spread of heterochromatin, whereas H2A.X is involved in DNA repair being recruited at double strand break (DSB) site, a process that requires the phosphorylation of the serine present at C-terminal motif SQEY/F. Interestingly, *T. gondii* has a variant of H2B, that has been named H2B.Z since it forms dimers mainly with H2A.Z. Double variant H2A.Z/H2B.Z nucleosome and H2A.X/H2Ba are not present in the same nucleosome as it was observed by ChIP-qPCR and ChIP-seq. These findings reveal that nucleosomal arrangements are not random in protozoa, highlighting their relevance in chromatin composition and regulation. H2A.Z and H2B.Z have shown to be highly acetylated at their N-terminal tails, a marker of active chromatin. *H2A.Z* and *H2B.Z* genes have shown to be essential. The over-expression of different H2B.Z mutants, that are unable to acetylate the N-tail, has shown little effect in tachyzoite replication rate but an important alteration in the differentiation process. On the other hand, proteomic analysis confirms the presence of gH2A.X in normal conditions suggesting that tachyzoites may be subjected to fork collapse and DSB, situations that activate the homologous recombination repair machinery. H2A.X is phosphorylated at its SQE motif by ATM kinase at the initial step of HRR pathway. Based on that, the impact on tachyzoite replication of CPT, which generates DSB during DNA replication, and KU55933, a highly specific ATM inhibitor was analyzed. Both, CPT and KU55933 produced a significant effect on parasite replication suggesting their inhibition effect may be blocking *T. gondii* DNA replication and/or activating cell cycle checkpoints either affecting *T. gondii* ATM directly or through HFF ATM inhibition. As expected, the combination of both

drugs generated a serious blocking of replication rates with a large number of tachyzoites that were not able to carry out the first event of replication. Taken together the results show that histone variants and their PTM are important epigenetic regulators in different processes of the parasite life cycle.

## NEW THERAPEUTIC STRATEGIES FOR ECHINOCOCCOSIS: MODIFICATION ON THE DRUG RELEASE TO INCREASE BIOAVAILABILITY AND EFFICACY

**MARÍA CELINA ELISSONDO**

*Laboratorio de Zoonosis Parasitarias, Facultad de Ciencias Exactas y Naturales*
*CONICET y Universidad Nacional de Mar del Plata, Argentina.*

Echinocccosis, also known as hydatid disease or hydatidosis, is a parasitic zoonoses caused by infection with the larval stage of the cestode *Echinococcus* spp. The World Health Organization has recently included human echinococcosis within the group of neglected tropical diseases, and recommends a veterinary public health strategy as part of an effective control approach.

Depending on different factors such as cyst number, size and location, viability status, the involved organ and location, the interaction between the expanding parasite and the adjacent host tissue and bacterial and fungal infection, there are different treatment and management options human echinococcosis.

In the last 30 years, an increase in the use of anthelmintic drugs for the medical treatment of echinococcosis was observed. The only two drugs licensed to date are the benzimidazole carbamate derivatives albendazole and mebendazole. Albendazole belongs to Class II of the biopharmaceutical classification system, with high permeability and low aqueous solubility. Approximately a third of the patients treated with benzimidazole drugs have been cured, 30–50% develop some evidence of a therapeutic response while between 20 and 40% of cases do not respond favourably. Therapeutic failures attributed to medical management of echinococcosis with albendazole have been primarily linked to the poor drug absorption rate (<5%) resulting in low drug level in plasma and cysts. On the other hand, the poor water solubility of albendazole offers only few formulation possibilities, limiting the administration routes.

Novel and improved therapeutical tools are needed in order to optimize treatment of human echinococcosis. Unfortunately, the pharmaceutical industry is not developing novel treatment options besides benzimidazoles against these neglected diseases. Novel chemotherapeutics have to be identified by one of the following strategies (1) *in vitro* and *in vivo* testing of broad-spectrum anti-infective drugs and drugs inhibiting proliferation of cancer cells; (2) new drug targets are being identified from the knowledge of the genome, transcriptome, proteome and metabolome of the parasite; (3) the use of pharmacotechnical strategies to optimize the use of existing drugs.

In this presentation, some of the new technologies applied to the experimental treatment of echinococcosis will be discussed. During the last 17 years, the Parasitic Zoonoses Research Group (Faculty of Natural and Exact Sciences, Mar del Plata National University) has been working in the experimental chemotherapy of hydatid disease. Since 2011, in a joint effort with the Laboratory of Pharmacotechnics (Faculty of Chemistry, National University of Cordoba), different albendazole drug delivery systems are being studied with the aim to improve the treatment against the murine model of echinococcosis. After evaluating the clinical and chemoprophylactic efficacy of a solid dispersion, nanocrystals and lipid nanoparticles, promising results have been obtained.

## PARASITE AND HOST GENETIC DIVERSITY IN TRANSPLACENTAL TRANSMISSION OF *TRYPANOSOMA CRUZI*

**NATALIA JUIZ, SILVIA LONGHI AND ALEJANDRO G. SCHIJMAN**

*LABMECH- INGEBI-CONICET, Buenos Aires, Argentina*

Congenital Chagas diseases has striking impact in public health, being partially responsible of the emergent urbanization of Chagas disease, not only in endemic countries but also in non-endemic continents due to migration movements.

The occurrence of this type of transmission in a variable percentage from 2 to 10% of descendants of infected pregnant women depends on the geographic área and is a product of the complex interaction of parasitic factors, such as the parasitic maternal load and the parasite genotype as well as genetic factors of the mammalian host.

The placenta is the key barrier the parasite must invade to reach the faetus so it is crucial to investigate the nature of placental factors to understand the mechanism

of transmission. In this context, we have started to study the existence of association between genetic host patterns and the likelihood of congenital transmission. We have explored associations between SNP polymorphism in placentary genes and congenital infection and have initiated transcriptomic analyses in murine model and human placentas.

**Analysis of SNPs in placentary genes.** A retrospective study was carried out using human DNA obtained from 217 blood samples from 101 congenitally infected and 116 non-infected children born to Chagasic mothers. We have analyzed the sequences of eleven SNPs located in 4 loci that encode placentary enzymes, described top lay a role in congenital transmission, namely: rs2014683 and rs1048988 in *ALPP gene coding for placental alkaline phosphatase*, rs11244787 and rs1871054 in *ADAM12*, rs243866, rs243865, rs17859821, rs243864 and rs2285053 de *MMP2* and rs3918242 and rs2234681 in *MMP9, all three genes with metalloprotease activities. SNP identification was achieved after developing Real Time PCR systems followed by* "High Resolution Melting analysis" for discrimination of allelic copies and by sequencing in a microsatellite variant.

An association was observed between SNPs in gene MMP2 and ADAM 12. Logistic regression analysis under dominant and recessive models revealed that for SNPs rs243866 and rs17859821 of gene MMP2, one copy of the mutant allele, "A" in both cases, was necessary to increase the risk of congenital infection. In the case of SNP rs2285053, both copies of the "T" variant are necessary. With respect to gene ADAM12, the frequency of SNPs rs11244787 and rs1871054 differed between infected and non-infected groups by comparing genotypic and allelic frequencies as well as after regression analysis under the dominant model. In the case of SNP rs1871054, a protective effect of the allele "T" was also observed under the recessive model. Following recent studies that proposed gene to gene interactions as involved in the pathogenesis of diseases, we carried out a multifactorial dimensional analysis to identify the existence of differential SNP-SNP interactions between infected and non-infected groups. Genotyping of five sites rs11244787, rs1871054, rs243866, rs17859821 and rs243864 would be good predictors of the susceptibility of congenital infection.

**Transcriptomic studies in placental genes.** We performed functional genomics by microarray analysis in C57Bl/6J mice comparing placentas from uninfected animals and from animals infected with two *T. cruzi* strains: K98, a clone of the non-lethal myotropic CA-I strain (TcI), and VD (TcVI), isolated from a congenitally infected patient. Analysis of networks by GeneMANIA of differentially expressed genes showed that "Secretory Granule" was a pathway down-regulated in both infected groups, whereas "Innate Immune Response" and "Response to Interferon-gamma" pathways were up-regulated in VD infection but not in K98. Applying another approach, the GSEA algorithm that detects small changes in predetermined gene sets, we found that metabolic processes, transcription and macromolecular transport were down-regulated in infected placentas environment and some pathways related to cascade signaling had opposite regulation: over-represented in VD and down-regulated in K98 group. We also have found a stronger placental tropism of the VD strain, by detection and quantification of parasite satellite DNA and 18s RNA, indicative of living parasites in the tissue sample.

Transcriptomic analysis by means of RNAseq are currently undergone to compare differentially expressed genes in human placentas from pregnant women with and without detectable parasitic burden measured by Real Time PCR.

Our study is the first one to describe genetic characteristics, such as SNPs in placental expressed genes and the genetic response of placental environment to *T. cruzi* infection and suggests the development of a strong immune response, parasite genotype-dependent, to the detriment of cellular metabolism.

## IMPACT OF PHARMACOKINETIC IN THE CLINICAL EFFICACY OF ANTHELMINTICS

**LUIS I. ALVAREZ**

*Centro de Investigaciones Veterinarias de Tandil (CIVETAN), FCV, UNCPBA-CICPBA-CONICET, Tandil, Argentina.*

Pharmacokinetics describes how the body affects the movement of a drug after its administration, and determines the drug concentration at the biophase (the site of action). Since the higher the concentration achieved at the tissue where the parasite is located, the greater the amount of drug reaching the target parasite receptor, pharmacokinetic is a key factor in anthelmintic efficacy. Understanding the mechanisms involved in drug access to the target parasite, together with drug pharmacodynamics, will enhance overall comprehension of anthelmintic drug activity. Two main issues are crucial to the comprehension of the process of drug accumulation into nematodes: (i) the oral versus transcuticular entrance routes, and (ii) identification of the main drug transport mechanism (active transport versus passive diffusion) involved in the transfer process. Lipophilicity and concentration of the active drug, the physico-chemical features of the parasite-surrounding medium, the structure of parasite's external surface, are among the factors affecting the transfer (diffusion) and accumulation of the active drug into the target parasite(s). Additionally, the particular mode of action of each compound will affect the onset and the character-

istic of the anthelmintic effect. Altogether, these different factors will determine the final anthelmintic activity. The pharmacokinetic "barrier" may explain many therapeutic failures observed in parasite control in both human and veterinary medicine, which in some cases have contributed to exposure of target parasites to subtherapeutic drug concentrations. The characterisation of drug concentration profiles in tissues of parasite location and within target parasites, and its relationship with the mode of action of each particular molecule provides a basis for understanding the differences in efficacy observed for the different chemical families. The entry of a drug into the parasite by a transtegumental/cuticular diffusion process may mainly depend on the diffusion surface, the concentration gradient across the membrane, the pH/pK relationship and the lipophilicity of the molecule. The amount of drug reaching the target nematodes is influenced by the drug concentrations in the tissues where the parasite is located. The higher concentrations measured in the gastrointestinal content, accounted for a greater amount of drug being measured within adult target worms recovered from treated animals. Drug absorption across nematode cuticle is restricted by lipid barriers in the hypodermis and collagen matrix. The rate of penetration across the cuticle depends mainly on lipophilicity, and, in the case of acidic or basic drugs, on the ionized and unionized (lipid-permeable) fractions of the drug, which is determined by the relationship between drug pK and pH of the aqueous environment within the cuticle. Although the oral route cannot be discarded, there is clear evidence that transcuticular diffusion is the common route of access for different anthelmintics in nematodes. A different situation occurs in trematode parasites such as *Fasciola hepatica*, in which the accumulated data confirm that oral ingestion is a main route of drug entry into adult liver flukes *in vivo* exposed to flukicidal drugs. Consequently, there is a strong relationship between blood concentration and flukicidal activity. This mechanism of drug entry has been observed under *in vivo* conditions for different flukicidal drugs such as triclabendazole, albendazole and closantel. The pharmacokinetic-pharmacodynamic relationship of anthelmintic drugs will be discussed based on different *in vivo* examples including trematode and nematode parasites.

## SAIC SYMPOSIUM VII

## ROLE OF MOLECULAR CHAPERONES IN HEALTH AND DISEASE

### RELEVANCE OF THE HSP90-IMMUNOPHILIN CHAPERONE SYSTEM IN THE REGULATION OF BASIC BIOLOGICAL PROCESSES IN HEALTH AND DISEASE

**G.I. MAZAIRA [1], C. DANERI [2], M.F. CAMISAY [1], N.R. ZGAJNAR [2], S.A. DE LEO [1], F. FEDERICCI [1], A.A. CAUERHFF [1], M.LAGADARI [2], A.G. ERLEJMAN [1], M.D. GALIGNIANA[1,2]**

*[1] Departamento de Química Biológica-Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina. [2] IBYME-CONICET, Buenos Aires, Argentina.*

Hsp90 is the major soluble protein of the cell. Most of the Hsp90 population is primarily cytoplasmic, and a small fraction is also nuclear and plays several structural and functional roles. In the cell, Hsp90 is a homodimer. Each protomer contains three flexibly linked regions-the N-terminal domain (or ATP-binding domain), the middle M-domain, and the C-terminal domain or dimerization domain. The latter shows a conserved MEEVD motif that serves as the docking site for Hsp90 co-chaperones via a tetratricopeptide repeat (TPR) clamp. Although in some studies is still under discussion what the real stoiquiometry of the interaction between Hsp90 dimers and TPR-domain co-chaperones is, there is a general consensus that in the cell it is likely that there is only one TPR protein bound per dimer of Hsp90. This early finding of our laboratory was subsequently validated by the regulatory action observed for several biological properties of Hsp90 client proteins due to the functional exchange of high molecular weight immunophilins such as FKBP51 and FKBP52 associated to Hsp90 via that TPR clamp. Here we will discuss the biological relevance of the Hsp90-immunophilin heterocomplex in the regulation of several biological models such as the steroid receptor function in health and disease, its involvement in cancer development and progression, the regulation of telomerase activity, the ability to promote the nuclear retention of transcription factors by nucleoskeleton arrangement, and its role in cell differentiation. In all of these basic biological situations, the properties shown by the Hsp90-immunophilin chaperone heterocomplex in the cell supports the existence of a single Hsp90-binding immunophilin bound per Hsp90 dimer, which can be dynamically exchanged by other TPR-domain proteins in a mutually exclusive fashion. In view of the number and relevance of signalling cascades and cellular events affected by this heterocomplex, the potential use of drugs with therapeutic purposes that may affect the organization and function of such protein arrangement is currently assayed in clinical trials.

## THE STRESS PROTEIN FKBP51 SHAPES ANTIDEPRESSANT PHARMACOLOGY AND LINKS TO A NOVEL DRUGGABLE ROUTE TO AUTOPHAGY

**GASSEN, N.C.[1]; STEPAN, J.[2]; BALSEVICH,G.[2]; HARTMANN,J.[2]; GENEWSKY, A.[2]; HAFNER, K.[1]; SCHMIDT, M.V.[2]; EDER, M.[2]; REIN, T.[1]**

*[1]Max Planck Institute of Psychiatry, Department of Translational Research in Psychiatry, Kraepelinstr. 10, Munich 80804, Germany; [2]Max Planck Institute of Psychiatry, Department of stress neurobiology and neurogenetics, Kraepelinstr. 10, Munich 80804, Germany*

FK506 binding protein 51 (FKBP51) is both regulator and target of the stress receptors. Genetic evidence implicated FKBP51 in stress-related psychiatric diseases such as depression and furthermore in the responsiveness to antidepressants. Since the molecular underpinnings remained elusive we set out to decipher intracellular pathways regulated by both antidepressants and FKBP51. Interaction analyses led to several convergently regulated molecular pathways in cells, mice and human. These pathways include GSK3beta, Akt1-Autophagy, and DNA methyltransferase 1 (DNMT1). Our studies characterize FKBP51 as remarkably versatile stress protein and scaffolder of multiple protein complexes. In patients suffering from depression, markers of these pathways predict clinical treatment response. To test whether these pathways might be causally involved in antidepressant action we used small molecules to address novel autophagy pathway components downstream of FKBP51. Some of them mimicked the action of antidepressants on autophagy pathway activity. Based in voltage-sensitive dye imaging, mini-EPSCs, and in vivo electrophysiological recordings, these compounds, acting downstream of FKBP51, also changed synaptic function similarly to antidepressants. Moreover, we observed antidepressant-like behavioral effects in mice. These compounds thus provide a novel route to autophagy, reveal a particular form of neuronal autophagy, and identify novel compounds for autophagic therapy in psychiatric and other diseases.

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## DIRECT TARGETING OF THE FKBP52 COCHAPERONE FOR THE TREATMENT OF CASTRATION RESISTANT PROSTATE CANCER

**N. GUY[1], H. XIE[2], J. CHAUDHARY[3], A. CHERKASOV[4] AND M.B. COX[1*]**

*[1]Border Biomedical Research Center and Department of Biological Sciences, University of Texas at El Paso, 500 W. University Ave., El Paso TX 79968, USA; [2]College of Pharmacy and Health Sciences, Texas Southern University, Houston, TX, USA; [3]Center for Cancer Research and Therapeutic Development, Clark Atlanta University, Atlanta, GA, USA; [4]The Prostate Centre, Vancouver General Hospital, Vancouver, Canada*

The FKBP52 cochaperone is a positive regulator of androgen (AR), glucocorticoid (GR) and progesterone receptor (PR) function and represents an attractive target for the treatment of castration resistant prostate cancer. Towards this end, we previously identified MJC13, which represents a first-in-class drug for targeting the regulation of AR by FKBP52 through binding a putative FKBP52 regulatory surface on AR. While the targeting of the FKBP52 regulatory surface on AR is a promising therapeutic strategy, we propose that the direct targeting of FKBP52 offers a number of advantages over MJC13 that would lead to a more potent and effective drug. Thus, we performed a high-throughput *in silico* screen of the ZINC database consisting of 20-million lead-like compounds. We identified GMC1 as the initial hit molecule with the most potent inhibition of FKBP52-mediated AR reporter expression. GMC1 effectively blocks AR, GR, and PR activity, blocks endogenous AR-mediated gene expression, and inhibits the proliferation of prostate cancer cell lines. As proof-of-concept we developed a soluble GMC1 co-solvent formulation and demonstrated that GMC1 prevents tumor growth and causes tumor recession in LNCaP and CW22Rv1 xenograft mouse models. These studies are at the forefront of an emerging concept to target novel AR co-regulators for the treatment of prostate cancer.

## (1073) THE PEPTIDYLPROLYL-ISOMERASE ACTIVITY OF FKBP52 IS REQUIRED TO ENHANCE NF-kB BIOLOGICAL ACTION

**S.A. DE LEO[1], M.F. CAMISAY[1], M. D. GALIGNIANA[1, 2], A.G. ERLEJMAN[1]**

*[1]departamento De Química Biológica / IQUIBICEN, FCEN, UBA, Buenos Aires, Argentina. [2]IBYME, Buenos Aires, Argentina*

Nuclear Factor κB (NF-κB) is a transcription factor that regulates the expression of genes involved in inflammation, cell cycle and cell death. NF-κB aberrant activation is relevant in chronic inflammatory diseases and cancer promotion. Inactive NF-κB is primarily cytoplasmic, and translocates to the nucleus upon activation. High MW FK506-binding proteins (FKBPs) are Hsp90-binding proteins first related to steroid receptor action. A signature property for FKBPs is the peptidylprolyl isomerase (PPIase) activity. Previously, we reported that FKBP51 and FKBP52 modulate canonical NF-κB (p65/p50) biological actions in an antagonistic fashion. In this work, we studied the contribution of FKBP52-PPIase activity on NF-κB biological action at different steps of its activation cascade, i.e. transcriptional activation by gene reporter assay, p65 nuclear relocalization by indirect immunofluorescence, and p65 phosphorylation at Ser536 by Western blot. NF-κB nuclear translocation was favored by overexpression of FKBP52 in HEK293T cells treated for 1 h with TNFalpha, while a mutant lacking enzymatic activity (FKBP52 F130Y) showed a decreased nuclear translocation. Accordingly, FKBP52 favored p65 phosphorylation as demonstrated by Western blot with a specific antibody and a band shift after alkaline phosphatase treatment. FKBP52 showed a strong stimulating effect on PMA-induced NF-κB transcriptional activation. The relevance of the PPIase activity for this effect was indirectly evidenced by the lack of stimulation in cells treated with the inhibitor FK506 (tacrolimus), and confirmed by overexpression of two inactive PPIase mutants. In contrast to FKBP52, its homologous partner FKBP51 impaired p65 nuclear translocation and transcriptional activity, the PPIase activity and its association with Hsp90 via the TPR domain being not required. In summary, FKBP52 activates NF-κB signalling cascade at various steps, its PPIase activity playing a key role in this regulation.

# SAIC-SAFE SYMPOSIUM VIII

# ONCO-HEMATHOLOGY AND INFLAMMATION

## IDENTIFYING THE MECHANISMS DRIVING INV(16) ACUTE MYELOID LEUKEMIA

**LUCIO H. CASTILLA**

*Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, USA*

The pathology and treatment of acute myeloid leukemia (AML) is largely defined by the mutation composition in their blast cells. The development of effective specific therapies in AML that target the function of driver oncoproteins is lacking. A fraction of AML cases have the chromosome 16(p16;q22) inversion, which encodes the leukemia fusion protein CBFβ-SMMHC. We have shown that this fusion protein is a founding event that creates a pre-leukemic myeloid progenitor cell, and drives leukemia development and maintenance in mouse models and patient-derived AML cells. Recently, we have developed a specific inhibitor of CBFβ-SMMHC function, AI-10-49, with excellent potential as a candidate for inv16 AML targeted therapy. This small molecule binds to the CBFβ portion of CBFβ-SMMHC with high specificity, inhibits its binding to RUNX1, and restores expression of RUNX1 target genes. AI-10-49 induces apoptosis of leukemia cells expressing CBFβ-SMMHC with high potency and specificity within 24 hours. AI-10-49 presents excellent pharmacokinetics and negligible toxicity in mice, and delays leukemia latency in a mouse model. Furthermore, this molecule shows selective activity in human inv16 AML blasts in vitro. Recent studies in my laboratory have combined pharmacologic and genomic (ATACseq, ChIPseq and RNA-seq) approaches in human AML cells complemented with genetic mouse models to understand the how AI-10-49 induces apoptosis in inv16 AML. These studies have identified key enhancers driven by RUNX1 and BRD4 that provide survival of leukemia cells, and that are repressed in the presence of AI-10-49. These studies will provide a rationale for developing effective combined targeted therapies for inv(16) AML.

# ONCOGENIC ENHANCER REARRANGEMENTS IN AML

**RUUD DELWEL**

*Department of Hematology, Erasmus University Medical Center, Rotterdam, The Netherlands.*

Introduction: The magnitude of data obtained through cancer genomics has greatly improved our insights into gene mutations and the effects of the mutated products on the biology of the disease, but it has yet to fulfil the promise of generating effective new therapies. Recent advances in molecular cancer research revealed entirely novel roles for epigenetic dysregulation of gene expression in the pathogenesis of acute leukemia. Since chromatin regulators are frequently amenable to small molecule inhibition, mutated regulatory regions are attractive targets for treatment of malignancies that are refractory to chemotherapy. In our program, we focus on advancing epigenetic approaches for a better understanding of leukemia dysregulation with the ultimate goal of pursuing unexplored epigenetic therapeutic opportunities.

An enhancer rearrangement uncovered in AML with inv(3)/t(3;3) applying functional genomics and genome editing.

Acute myeloid leukemia (AML) with chromosomal rearrangements inv(3) or t(3;3) is characterized by overexpression of the proto-oncogene EVI1 and clinically by an extremely poor response to therapy. We recently uncovered the molecular basis of EVI1 deregulation of AML subtype with inv(3)/t(3;3). High-resolution mapping of chromosomal breakpoints by 3q-seq revealed a breakpoint-free segment of 18 kb size near GATA2 (3q21) relocating to the EVI1 locus (3q26) in all analyzed cases. 4C-seq was carried out to study the three-dimensional chromatin environment of the EVI1 promoter, which revealed a contact hotspot of 9 kb within this commonly rearranged segment. ChIP-Seq analysis for enhancer-associated p300-binding confirmed a p300 interacting region in the center of the 9kb chromatin segment that formed a complex with EVI1 promoter. Excision of the ectopic enhancer element in an inv(3) cell line model (MUTZ-3), using CRISPR/Cas9 genome editing, abrogated EVI1 transcription and led to a profound reduction in cell viability, higher rates of apoptosis, along with increased maturation of the AML cells toward monocyte/macrophage phenotype. Exactly the same effects were observed upon EVI1 knock-down in these cells. Thus, aberrant EVI1 expression was caused by the reallocation of an enhancer, which caused uncontrolled expression of the transforming EVI1 gene.

We found that the identified enhancer element was a constituent of the of the GATA2 regulatory domain located at 3q21. RNA-seq and qPCR analysis confirmed reduced and monoallelic GATA2 expression only from the remaining normal chromosome 3 allele in inv(3)/t(3;3) AMLs, compared to control AMLs. Thus a single oncogenic enhancer rearrangement causes concomitant EVI1 and GATA2 deregulation in inv(3)/t(3;3) AMLs. In 20% of the inv(3)/t(3;3) AML patients, mutations were found in the coding region of GATA2 in the expressed the allele that was expressed. Thus, in those cases, no wild type GATA2 was present in those cells, emphasizing that aberrant GATA2 expression and function plays an important role in leukemia onset, development and/or maintenance in inv(3)/t(3;3) AML.

EVI1 is activated by an oncogenic super enhancer in AML with inv(3)/t(3;3)

We discovered that the GATA2 enhancer which translocates to EVI1, turns into a so-called oncogenic super-enhancer or stretched enhancer (OSE) in AML with inv(3)/t(3;3). The newly derived oncogenic EVI1-OSE is hyper-sensitive to pharmacologic BET-inhibitors, whereas the non-rearranged enhancer near GATA2 is insensitive to this compound. The mechanism of action of OSEs, why they are hyper-responsive to BET-inhibitors and which other components are essential for their activity is not understood. Nevertheless the lack of understanding, are BET-inhibitors currently applied in Phase I/II clinical trials, in particular for patients with acute myeloid leukemia (AML) that are refractory to chemotherapy. These patients are not selected based on prior knowledge about their sensitivity to those drugs or whether OSE-activated disease genes play a role in leukemic transformation of these patients. Since, normal super enhancers (SEs) are also sensitive to BET-inhibitors, there are question marks about the specificity of these compounds. An important difference between SEs and OSEs is that SEs are precisely regulated and can be switched on or off during cellular development, whereas OSEs are constitutively active. We hypothesize that altered regulation of the OSEs is caused by critical regulators of transcription and involves specific domains in the mutated OSE.

Conclusion: Our studies show that altered enhancer function is crucial in leukemia development in at least a portion of human AMLs and that molecular understanding of defective transcriptional control may provide leads for hypothesis driven targeting of cancer.

## IDENTIFYING MECHANISMS OF GLUCOCORTICOID RESISTANCE IN RELAPSED PEDIATRIC T-ALL

**JUSTINE E. RODERICK, KAYLEIGH GALLAGHER, KATHERINE M. TANG, OLIVIA KUGLER-UMANA, JULIE ZHU, MICHAEL R. GREEN AND MICHELLE A KELLIHER**

*Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester MA 01605, USA*

While great strides have been made in the improvement of outcome for newly diagnosed pediatric acute lymphoblastic leukemia (ALL) patients, prognosis for relapsed leukemia patients remains poor. The synthetic glucocorticoid (GC) dexamethasone is part of the standard treatment for pediatric ALL and patient response to glucocorticoid treatment has proved to be a reliable prognostic indicator[1–3]the largest multicenter trial of the Berlin-Frankfurt-M\u00fcnster (BFM. Identifying the biological pathways responsible for glucocorticoid resistance may reveal novel therapeutic targets to prevent and treat relapsed ALL. Although genomic analyses of relapsed patients and matched diagnosis-relapse patient pairs have begun to define the genomic landscape of relapsed disease [4–6]but the prognosis is dismal for the minority of patients who relapse after treatment. To explore the genetic basis of relapse, we performed genome-wide DNA copy number analyses on matched diagnosis and relapse samples from 61 pediatric patients with ALL. The diagnosis and relapse samples typically showed different patterns of genomic copy number abnormalities (CNAs, discerning "driver from passenger" genetic lesions remains challenging. To identify glucocorticoid resistance genes in an unbiased, high-throughput manner, we conducted a genome wide, survival based, shRNA screen in dexamethasone sensitive murine T-ALL cells. Our preliminary data identify several hundred genes capable of mediating GC resistance, including several known GC resistance genes *Nr3c1*, *Rcan1, Btg1* and *Mllt10*, thereby validating our experimental approach. Candidate genes identified in the screen including *EP300* (p300), *GATA3* and *IKZF1* are known leukemia suppressors in pediatric ALL and the EP300 paralog CREBBP and IKAROS have been linked to GC resistance[5]but the biological determinants of treatment failure remain poorly understood. Recent genome-wide profiling of structural DNA alterations in ALL have identified multiple submicroscopic somatic mutations targeting key cellular pathways, and have demonstrated substantial evolution in genetic alterations from diagnosis to relapse. However, DNA sequence mutations in ALL have not been analysed in detail. To identify novel mutations in relapsed ALL, we resequenced 300 genes in matched diagnosis and relapse samples from 23 patients with ALL. This identified 52 somatic non-synonymous mutations in 32 genes, many of which were novel, including the transcriptional coactivators CREBBP and NCOR1, the transcription factors ERG, SPI1, TCF4 and TCF7L2, components of the Ras signalling pathway, histone genes, genes involved in histone modification (CREBBP and CTCF, indicating that suppressor genes involved in human leukemia and GC resistance are identified in our mouse screen. Consistently, we found the expression of several screen hits significantly decreased and/or mutated in relapse patient samples. Novel dexamethasone resistance genes identified in the screen interfere with GC-induced transcription (*Stat3, Rfx3, Sox6, Ncor2*), promote pluripotency (*Esrrb, Sox2)* or stimulate cAMP signaling (*Adcy3, Adcy9, Gnas, Creb1)*. Silencing of these genes in multiple mouse T-ALL cell lines has no detectable effects on leukemic growth/survival *in vitro,* but confers resistance to dexamethasone treatment *in vitro* and *in vivo*. Moreover, we show that silencing of some candidate dexamethasone resistance genes accelerates leukemogenesis *in vivo,* demonstrating that leukemia suppressor genes were identified. Effect(s) of silencing or inhibiting these novel dexamethasone resistance genes/pathways in human T-ALL cell lines, primary patient samples and xenografts will be discussed. We predict that targeting these dexamethasone resistance pathways may re-sensitize relapse pediatric T-ALL cells to dexamethasone and/or contribute to more effective patient stratification to prevent relapse and induction failure.

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## NEGATIVE REGULATION OF THE IMMUNE RESPONSE

**CARLA ROTHLIN**

*HHMI Faculty Scholar, Associate Professor Immunobiology and Pharmacology,*
*Yale School of Medicine, New Haven, USA*

The innate immune response of dendritic cells (DCs) and others entinel cells functions as both the first line of defense against pathogens and also as the initiating trigger for T-cell-mediated adaptive immunity. These fundamental activities not with standing, DC activation must be tightly regulated. While reduced DC function leads to increased susceptibility to infections, unrestrained, overactive DC responses can lead to allergy, autoimmunity,

chronic inflammatory disease, and other pathological conditions. We have found that the TAM receptor tyrosine kinases, TYRO3, AXL and MERTK, are potent negative regulators of the immune response in DCs. I will present new findings that's how that TAM signaling in DCs is triggered by cells of the adaptive response with which DCs interact with. I will also discuss the specificity of different TAM receptors in the regulation of the immune response.

## SAIC-SAI-SAFE SYMPOSIUM IX

## NEW MOLECULAR TARGETS FOR ONCOLOGICAL THERAPY

### PERSONALISED MEDICINE IN MELANOMA PATIENTS

**ROMINA GIROTTI**

*Instituto de Biología y Medicina Experimental, IBYME-CONICET, Argentina*

BRAF is mutated in about 50% of human melanomas and treatment with BRAF or MEK inhibitors have resulted in increased progression-free and overall survival in melanoma patients. However, the majority of patients relapse after a relatively short period of disease control. Furthermore, after treatment with targeted therapy, most patients derive little benefit from immune checkpoint inhibitors. Resistance to targeted agents is driven by several mechanisms, so selecting second line therapies is challenging. Current advice includes the option to continue treatment beyond progression, but it is unclear how to select the patients that will benefit from this, so detecting disease progression early and elucidating the mechanisms of resistance to therapy will help optimise the clinical care of these patients. Treatment options are also needed for the ~50% of melanoma patients who are BRAF wild-type.

We developed two novel compounds that target mutant BRAF and wild-type CRAF. Our compounds inhibited the growth of melanoma cells that were resistant to BRAF selective inhibitors. ERK pathway reactivation is responsible for resistance to BRAF targeted therapies in ~60% of the patients and in ~25% of patients resistance is driven by acquisition of mutations in NRAS. We show that our compounds inhibited the growth of melanoma cells that were resistant to BRAF-selective inhibitors due to pathway reactivation mediated by different mechanisms. We show that the drugs were active against patient derived xenografts (PDXs) from patients with acquired or intrinsic resistance to BRAF-selective inhibitors and in whose tumors resistance was associated with ERK pathway reactivation. Further, our compounds are active in a PDX from a patient whose tumor developed acquired resistance to a combination of a BRAF-selective plus a MEK inhibitor and associated with acquisition of an NRAS mutation. Thus, our panRAF inhibitors can inhibit melanomas with different mechanisms of acquired or intrinsic resistance to BRAF-selective and BRAFselective/MEK inhibitor combinations, potentially providing first-line treatment for naïve patients and second-line treatments for a range of relapsed patients (Girotti et al, Cancer Cell, 2015).

Moreover, we used whole exome sequencing (WES) to provide insight into the mechanisms of resistance to BRAF inhibition and identify new therapeutic strategies for BRAF wild-type melanomas. We present the case of a patient that was wild-type for V600 BRAF, but carried HRAS and Rb1 mutations, allowing us to predict that the patient's tumour would be sensitive to the combination of a MEK inhibitor plus paclitaxel and we validated this therapy in a xenograft derived from the patient (PDX). Thus we show that genome analysis can be used to develop novel hypothesis-driven therapeutic strategies for patients and we show that these treatments can be validated in the patients' PDXs. Finally, we describe the use of circulating tumour DNA (ctDNA) as a predictive biomarker of response to therapy and as a powerful approach to reveal and then monitor mechanisms of resistance. In summary, we are implementing a powerful combination of techniques for personalised medicine to improve clinical management of BRAF wild-type and BRAF mutant melanoma patients (Girotti et al, Cancer Discovery, 2016).

### VOLTAGE DEPENDENT ANION CHANNEL, A MITOCHONDRIAL ANTI-PROLIFERATIVE SWITCH

**EDUARDO N. MALDONADO**

*Department of Drug Discovery & Biomedical Sciences and Hollings Cancer Center*
*Medical University of South Carolina, Charleston, SC 29425, USA*

Otto Warburg in the early 20$^{th}$ century described a metabolic tumor phenotype characterized by enhanced glycolysis and suppression of mitochondrial metabolism even in the presence of physiological levels of oxygen. Warburg also erroneously postulated that permanent defective respiration originates cancer. Further inves-

tigations showed enhanced glycolysis and functional mitochondria in nearly all tumors and cancer cell lines. Differentiated cells produce about 95% of the total ATP by mitochondrial oxidative phosphorylation and the remaining 5% by aerobic glycolysis. By contrast in cancer cells, glycolysis contributes 20-90% of total ATP production. A highly glycolytic phenotype has been associated with a high rate of cell proliferation and considered an indicator of malignancy. The "glucose avidity" of tumors is the foundation for the positron emission tomography (PET) of the $^{18}$fluorodeoxyglucose to diagnose primary tumors, recurrences and metastasis. The advantage of the pro-proliferative Warburg phenotype for dividing cells is that the incomplete breakdown of glucose by glycolysis, although energetically less efficient than oxidative phosphorylation, provides carbon backbones for biomass generation (lipids, proteins, and nucleic acids). Biosynthesis of macromolecules is also contributed by intermediaries generated in the mitochondrial matrix.

In cancer cells, the interdependent "glycolytic" and "mitochondrial" metabolic compartments are separated by the mitochondrial outer membrane (MOM). The MOM is a functional barrier containing the voltage-dependent anion channel (VDAC) that comprises 3 isoforms in humans, VDAC1, VDAC2 and VDAC3. Flux of metabolites entering mitochondria including respiratory substrates, ADP and Pi cross the MOM through only one channel, VDAC. Once inside the matrix, respiratory substrates fuel the Krebs cycle generating mostly NADH that enters the electron transport chain (ETC). The ETC transfers electrons to the final acceptor $O_2$, pumps protons to the intermembrane space, and generates reactive oxygen species (ROS). Protons in the intermembrane space create a negative potential in the mitochondrial matrix (mitochondrial membrane potential, $\Delta\Psi$) and a proton-motive force utilized by the ATP synthase to generate ATP.

VDAC opening operates as a "master key" that "seal-unseal" mitochondria to modulate mitochondrial metabolism, ROS formation and the intracellular flow of energy. Tumors are metabolically flexible and can switch the bioenergetics phenotype from glycolytic to oxidative and vice versa in response to different stimuli. A predominantly oxidative metabolism characteristic of differentiated cells leads to a high cytosolic ATP/ADP ratio that inhibits glycolysis. A low ATP/ADP ratio, essential to maintain enhanced glycolysis independently of other pro-glycolytic variables, requires a partial or complete suppression of mitochondrial metabolism. VDAC, initially considered an all-time open channel, is actually regulated by several factors including free tubulin. We showed that high free tubulin in cancer cells decreases mitochondrial $\Delta\Psi$ by limiting ingress of respiratory substrates and ATP. Dimeric $\alpha$-$\beta$ tubulin also decreases conductance of VDAC inserted in lipid bilayers. We also showed that VDAC knockdown decreases mitochondrial metabolism. Our findings led to the hypothesis that VDAC closing by free tubulin contributes to the suppression of mitochondrial metabolism in the Warburg effect.

The VDAC-tubulin interaction is a potential pharmacological target to increase mitochondrial metabolism, promote ROS formation and revert the Warburg effect. We found that erastin, a small molecule that kills cells engineered to harbor a $RAS^{v12}$ mutation, antagonizes the inhibitory effect of tubulin on VDAC. We also identified lead "erastin-like" compounds in a cell-based high throughput screening of a chemical library of 50,000 small molecules. Both erastin and lead compounds follow a "metabolic double hit model" characterized by induced oxidative stress and decreased glycolysis (anti-Warburg). Blockage of the inhibitory effect of tubulin on VDAC increases mitochondrial metabolism and ROS formation and activates the stress kinase c-Jun N-terminal kinase (JNK) leading to mitochondrial dysfunction, bioenergetics failure and cell death. VDAC opening is not expected to kill every metabolically heterogeneous tumor cell. Cells that survive the initial ROS-dependent hit may eventually take the second hit, the reversal of the Warburg phenotype. VDAC-opening reverts the Warburg phenotype by switching cells to an oxidative metabolism as evidenced by the decrease in lactic acid release after erastin/erastin-like compounds. In summary, VDAC-tubulin antagonists are a novel group of drugs that target a molecular interaction with global effects on cancer cell bioenergetics and promote a two-hit effect, damage by oxidative stress and reversal of the pro-proliferative Warburg phenotype.

Grants: R01CA184456 (NCI) and COBRE Project GM103542 to ENM.

## REPROGRAMMING OF TUMOR CELLS WITH THE MIR 302/367 CLUSTER SUPPRESSES TRANSFORMATION PHENOTYPES

**CHUL MIN YANG AND BERND GRONER**

*Georg Speyer Haus, Institute for Tumor Biology and Experimental Therapy, Frankfurt am Main, D-60596, Germany*

Cellular transformation is initiated by the activation of oncogenes and a closely associated developmental reprogramming of the epigenetic landscape. Transcription factors, regulators of chromatin states and microRNAs influence cell fates in development and stabilize the phenotypes of normal, differentiated cells and of cancer cells. The miR-302/367 cluster, predominantly expressed in human embryonic stem cells (hESs), can promote the cellular reprogramming of human and mouse cells and contribute to the generation of iPSC. We have used the epigenetic reprogramming potential of the miR-302/367 cluster to "deprogram" tumor cells, that is, shift their gene

expression pattern towards an alternative program associated with more benign cellular phenotypes. Induction of the miR-302/367 cluster in extensively mutated U87MG glioblastoma cells drastically suppressed the expression of transformation related proteins, for example, the reprogramming factors OCT3/4, SOX2, KLF4 and c- MYC, and the transcription factors POU3F2, SALL2 and OLIG2, required for the maintenance of glioblastoma stem-like tumor propagating cells. It also diminished PI3K/AKT and STAT3 signaling, impeded colony formation in soft agar and cell migration and suppressed pro-inflammatory cytokine secretion. At the same time, the miR 302/367 cluster restored the expression of neuronal markers of differentiation. Most notably, miR-302/367 cluster expressing cells lose their ability to form tumors and to establish liver metastasis in nude mice. The induction of the miR-302/367 cluster in U87MG glioblastoma cells suppresses the expression of multiple transformation related genes, abolishes the tumor and metastasis formation potential of these cells and can potentially become a new approach for cancer therapy.

***Reference***

Expression of the miR-302/367 cluster in glioblastoma cells suppresses tumorigenic gene expression patterns and abolishes transformation related phenotypes. Chul Min Yang, Tomohiro Chiba, Boris Brill, Natalia Delis, Viktoria von Manstein, Vida Vafaizadeh, Thomas Oellerich and Bernd Groner. Intern. J. Cancer. 137, 2296-309 (2015)

## SAIC SYMPOSIUM X

## REPRODUCTIVE HEALTH

### MATERNAL DIABETES: MECHANISMS INVOLVED IN INTRAUTERINE PROGRAMMING OF METABOLIC, CARDIAC AND REPRODUCTIVE ANOMALIES

**ALICIA JAWERBAUM**

*Laboratorio de Reproducción y Metabolismo. CEFYBO-CONICET. Facultad de Medicina. Universidad de Buenos Aires.*

Maternal diabetes increases the risks for embryo resorption and malformations, feto-placental impairments and perinatal morbidities. Besides, the offspring have increased risks of metabolic and cardiovascular diseases in their adult life, as a result of an adverse process of intrauterine programming that is still poorly understood. Animal models of diabetes and pregnancy are valuable tools to improve the understanding of the mechanisms of induction of these alterations. In our laboratory, using a model of mild pregestational diabetes, we found that diabetic rats mated with control males lead to offspring that have increased markers of a pro-oxidant/pro-inflammatory state in their hearts from the neonatal stage. In the offspring from thesediabetic rats, increases in circulating lipid concentrations are evident from day 21 of age and increased circulating glucose concentrations are evident from the fifth month of age. Moreover, if normoglycemic three month-old offspring of pregestational diabetic rats are mated with control males, the pregnant rats develop gestational diabetes (GDM). At term, GDM fetuses are overweight and GDM placentas show reduced peroxisome proliferator activated receptors (PPARs) and increased mechanistic target of rapamycin (mTOR) signaling. PPARs and mTOR are nutritional regulators respectively activated by unsaturated fatty acids and amino acids, and respectively involved in anti-inflammatory and growth pathways. We found that PPARs can be activated in fetuses and placentas by supplementation of the maternal diet with oils rich in unsaturated fatty acids. Moreover, we found that these diets also have benefits in the offspring, as shown by reduced oxidative/inflammatory markers and reduced lipid content and peroxidation in the heart of the offspring of pregestational diabetic animals. These effects were similar to those found with the maternal administration of mitochondrial antioxidants, highlighting the relevance of oxidative stress in the intrauterine programming of offspring diseases in maternal diabetes. Besides, diets enriched in PUFAs in the pregnancy of pregestational diabetic animals (F0 generation)regulate placental PPAR and mTOR signaling, reduce feto-placental pro-oxidant/pro-inflammatory markers and prevent fetal overgrowth in the offspring that develop GDM during their pregnancy (F1). Our results suggest that impaired PPAR pathways are involved in the intrauterine programming of alterations in the heart, lipid metabolism, placental signaling and fetal growth in the offspring of pregestational diabetic rats, and that maternal supplements with oils enriched in PPAR ligands, possibly reducing the pro-oxidant/pro-inflammatory intrauterine environment, can prevent these alterations.

## THE IMPACT OF THE SERUM OF PATIENTS WITH ENDOMETRIOSIS ON RECONSTITUTED 3D-ENDOMETRIAL TISSUE: EVALUATION OF INFLAMMATORY CYTOKINES

**CAROLINE BORGATO[1]; ALINE LORENZON-OJEA[1]; ELAINE CARDOSO[1]; TATIANA BONETTI[2]; EDUARDO L. A. DA MOTTA[3]; PAULO C. SERAFINI[3]; VANESSA FREITAS[1]; ALEXANDRE BORBELY[1,4]; MAURICIO S. ABRAO[5]; LIDIA H. J. MYUNG[5]; AND ESTELA BEVILACQUA[1]**

*[1]Instituto de Ciências Biomédicas, Universidadede São Paulo, SP; [2]Depto de Obstetrícia, Universidade Federal de São Paulo, SP; [3]Depto de Ginecologia, Universidade Federalde São Paulo, SP; Centro de Medicina Reprodutiva Huntington, São Paulo, SP; [4]Institutode Biologia e Ciências da Saúde, Universidade Federal de Alagoas, AL; [5]Faculdade de Medicina da Universidade de São Paulo, SP, Brasil.*

Background - Endometriosis is a chronic inflammatory disease characterized by the presence and growth of ectopic endometrial tissue. It affects 10% to 15% of women at reproductive phase and is associated with severe inflammationand infertility. Altered profile of systemic immunological factors seems to be the primary factor associated with subfertility in these patients, regardless the severity of the endometriotic injury. Objectives: Using a three-dimensional (3D) culture of partially reconstituted endometrium, we explored the possibility of factors present in the plasma of endometriotic women affect the endometrial profile of cytokines determining changes that can impair the fertility. Material and Methods: Samples were collected after written informed consent was obtained (Research Ethics Committee in Human Beings, USP, no. 692457). Endometrial biopsies (n=9) and plasma (n=31) were collected from healthy women at the Huntington Reproductive Medicine, SP. Plasma from endometriotic patients (with estrogen/progestin hormonal therapy, n=10and untreated n=5) was obtained from this clinic and from the Medical School Hospital, University of São Paulo, SP. The biopsies were digested with collagenase II/DNAse I and filtered for retention of the endometrial glands.CD105magnetic microbeads (MACS)were used for positive selection of endothelial cells from filtered preparations. Adhered cells (fibroblastsand decidual cells) that passed through the column were ressuspended in DMEM / F12 medium.To construct the 3D environment, 0.1 x $10^6$ stromal cells in supplemented 199 medium were added to a mixture of extracellular matrix components (fibronectin and, collagen V, I and III) and placed in 48-well plates. After 12 hours in culture (37°C with 5% $CO_2$), endothelial cells were added (0.1 x $10^6$ cells) on the surface of the culture. After 24h, medium was replacedby one containing 20%serum from healthy or endometriotic women as follow (n = 3-8): i) Serum of healthy patients; ii) Serum of patients with endometriosis; iii)Serum of patients with endometriosis, which received hormonal therapy. The system remained in culture for additional 24 and 48 h. Cells were then homogenized for inflammatory cytokines evaluations through cytometric bead array, according to the manufacturer's instructions. Results and Conclusions: This study showed that the endometrium is not responsive to the serum of patients with endometriosis treated with hormonal therapy; expression levels of inflammatory cytokines did not show changes. The use of serum of women with endometriosis without treatment induced a significant increase of inflammatory cytokines (IFN-gamma, TNF-alpha, and IL-2) produced by the endometrium, suggesting that the absence of therapy in these women may be a key factor compromising the physiology of the endometrium andits reproductive function.In conclusion, this study showed an imbalance in cytokine expression in 3D-endometrial co-cultures treated with serum from endometriotic patients emphasizing the ability of the uterus to respond and contribute to a systemic inflammatory environment with cytokine production, even when distant from the endometriotic lesion. Immunological mediators as inflammatory cytokines may alter tightly regulated crucial gene expression, which may be an additional aspect for infertility in these women.

Financial Support: FAPESP, CNPq and CAPES.

## SPHINGOLIPIDS AND LOW-INTENSITY LASERS: ¿POSSIBLE STRATEGIES FOR ONCOFERTILITY OR SCIENCE FICTION?

**FERNANDA PARBORELL**

*Instituto de Biología y Medicina Experimental (IBYME)-CONICET, Buenos Aires, Argentina*

Oncofertility is an emerging interdisciplinary field that involves the study and development of new preventive and protective measures to reduce the impact of cancer treatments on reproductive health. Due to the increased survivorship of children and reproductive age patients treated with cancer thanks to the advances in anti-tumoral therapies, it is essential to understand its effects on future life quality and seek new techniques focused on preserving fertility. For example, in Argentina, breast cancer represents the highest incidence cancer in women, with a rate of 71 cases per 100,000 women. However, mortality rates from breast cancer have declined steadily and significantly since 1996. It is worth mentioning that,at a reproductive level, in patients older than 20 years who have received

anti-tumoral treatmentsthe rate of amenorrhea is 80%, the rate of premature ovarian failure (POF) is 90% and only 5-10% of these patients achieve spontaneous pregnancies. POF is a multi-disorder characterized by the disappearance or dysfunction of the ovarian follicles in women under 40 years. These patients present amenorrhea, hypoestrogenism and high levels of gonadotropins. POF affects about 1-2% of women under 40 years old and 0.1% of women under 30 yearsold and its causes can be grouped into: genetic, immune, infectious and iatrogenic (chemotherapy, radiotherapy) causes. In particular, POF can be consequence of treatment with chemotherapeutic drugs and/or radiation, with alkylating agents and radiotherapy being the most damaging agents to the ovarian reserve, and thus lead to infertility. Currently, treatments for POF consist mainly of hormone replacement therapy and oral contraceptives but are not fully effective. In addition, there are various options to preserve fertility (GnRH agonists, cryopreservation of oocytes, embryos and ovarian tissue). In our laboratory, we propose two new strategies to protect the ovary and restore fertility in patients who are diagnosed with cancer and undergoing chemotherapy treatment: 1) Local administration of ceramide-1-phosphate sphingolipid (C1P) (pharmacological strategy) 2) Local application of low intensity laser (LBI) (photobiomodulation).We have observed that both C1P and LBI are able to preserve ovarian reserve in a POF model induced by cyclophosphamide in mice. Finally, given the increased survival of cancer patients of reproductive age, it is necessary to develop effective, safe and inexpensive strategies to protect the ovary and preserve fertility, without neglecting the social, ethical and legal aspects, especially now that Argentina has a national law that provides medical assistance to those patients facing this difficult situation.

## MOLECULAR MECHANISMS ASSOCIATED WITH THE FERTILIZING CAPACITY OF MAMMALIAN SPERM

**MARIANO G. BUFFONE**

*Instituto de Biología y Medicina Experimental-CONICET, Buenos Aires, Argentina.*

Mammalian spermatozoa are not able to fertilize oocytes immediately after ejaculation; they must first undergo a complex process called capacitation in the female reproductive tract or *in vitro*. These changes include the development of hyperactivated motility and the ability to undergo acrosomal exocytosis (AE) in response to specific stimuli. AE is essential for fertilization. Mice and men that produce sperm lacking acrosomes are sterile. The occurrence of AE allows IZUMO1, a protein that is essential for sperm-egg fusion, to relocalize to the equatorial region of mouse sperm head. Not long ago, it was broadly accepted that sperm undergo AE upon interaction with the zona pellucida (ZP) of the egg, and many of the advances in our knowledge of this process were derived from in vitro studies using solubilized ZP. However, recent evidence acquired using transgenic mice that produce sperm carrying enhanced green fluorescent protein (EGFP) in the acrosome and Ds-Red2 red fluorescence in the mitochondria of the flagellar midpiece suggest that sperm binding to the ZP is not sufficient to induce AE. Real-time imaging of in vitro fertilization of cumulus-oocyte complexes (COCs) showed that most fertilizing sperm undergo AE before contacting the ZP. Therefore, the aim of this study was to determine physiological sites of AE by using double transgenic mouse sperm, which carried EGFP in the acrosome and DsRed2 fluorescence in mitochondria. Using live imaging of sperm during in vitro fertilization of cumulus-oocyte complexes, it was observed that most sperm did not undergo AE. Thus, the occurrence of AE within the female reproductive tract was evaluated in the physiological context where this process occurs. Most sperm in the lower segments of the oviduct were acrosome-intact; however, a significant number of sperm that reached the upper isthmus had undergone AE. In the ampulla, only 5% of the sperm were acrosome-intact. These results support our previous observations that most of mouse sperm do not initiate AE close to or on the ZP, and further demonstrate that a significant proportion of sperm initiate AE in the upper segments of the oviductal isthmus.

## SAIC SYMPOSIUM XI

## VIROLOGY AND DISEASE

# HERPESVIRAL ONCOGENESIS AND EFFECTS ON B CELL REPERTOIRE

**ETHEL CESARMAN**

*Weill Cornell Medicine, New York, USA*

Since the discovery of Epstein-Barr virus (EBV) in Burkitt lymphoma over 50 years ago, only one other herpesvirus, namely Kaposi's sarcoma-associated herpesvirus/human herpesvirus-8 (KSHV/HHV-8), has been shown to be a direct cause of cancer in humans. EBV causes a number of B cell lymphomas, carcinomas, and rarely leiomyosarcomas. KSHV is the infectious cause of three human malignancies, Kaposi Sarcoma (KS), an endothelial tumor, as well as Primary Effusion Lymphoma (PEL) and the plasma cell variant of Multicentric Castleman Disease (MCD), two B-cell lymphoproliferative diseases. The frequency of lymphoid malignancies related to infection by one of these two herpesviruses is greatly increased in individuals with immunodeficiency, whether primary or acquired, for example, as a consequence of HIV infection and AIDS or in the case of therapeutic immunosuppression for organ transplantation.

Understanding the causal associations of EBV and KSHV with certain cancers has allowed more accurate diagnosis and classification. Our current understanding indicates that EBV and KSHV contribute to lymphomagenesis by affecting genomic stability and by subverting the cellular molecular signaling machinery and metabolism to avoid immune surveillance and enhance tumor cell growth and survival. A deeper understanding of specific mechanisms by which EBV and KSHV cause cancer has been acquired over the past years, in particular with respect to viral protein interactions with host cell pathways, and microRNA functions. Specific therapies based on knowledge of viral functions are beginning to be evaluated, mostly in preclinical models.

KSHV is able to induce inflammation and angiogenesis when infecting endothelial cells, explaining its unique ability to cause KS. However, traditionally it has been challenging to do sophisticated studies in the context of full viral infection to assess specific KSHV properties because this virus does not infect cells or replicate efficiently *in vitro*. Recent advances have allowed us to use BAC systems to mutate specific viral genes, produce recombinant virus with selection markers, and assess the consequences of infection of different cell types. Using this technology, we have examined the effects of infection of primary B cells. We have known for almost 20 years that in MCD, KSHV infection of B lymphocytes is almost exclusively restricted to lambda light chain (IgL) expressing cells, and kappa light chain (IgK) cells that are KSHV positive are almost never observed. This specific association of KSHV biology with IgL has been a longstanding conundrum in the field given that IgL and IgK lymphocytes should be physiologically indistinguishable. In order to explore early pathogenic events during KSHV infection of lymphocytes, we have developed a model using *de novo* KSHV infection and maintenance of primary naïve B lymphocytes from human tonsil. Although at early stages of infection, we observe equal infection of B cells bearing immunoglobulin lambda (IgL) and kappa (IgK) light chains, the IgK positive cells are lost over time in the infected cultures. Experiments in which IgK and IgL naïve cells were sorted and infected separately reveal that KSHV infection induces IgK B cells to become IgL positive via a IgL/IgK double-positive intermediate. Consistent with these results, we observe polyclonal IgL genomic rearrangements in KSHV-infected IgK B cells by PCR, lambda light chain expression by RT-PCR and expression of the cellular V(D)J-recombination proteins Recombinase Activating Genes 1 and 2 (RAG1 and RAG2). Taken together our data demonstrates that KSHV infection of mature B lymphocytes causes re-induction of V(D)J-recombination in mature B lymphocytes, a process called B cell receptor (BCR) revision. Aside from providing a new and intriguing explanation for IgL restriction in KSHV infection, the potential implications of these results are far-reaching. BCR revision is associated with the induction of autoimmunity and these results could represent the first demonstration of a mechanism by which a lymphotropic human virus directly induces an autoimmune state. Autoimmunity is one of the diagnostic criteria for multicentric Castleman's disease, a KSHV-associated lymphoproliferative disorder, and moreover, autoimmune inflammation resulting from KSHV-mediated BCR revision could represent a critical driver of KSHV-associated lymphoma.

## ANTIVIRAL, ANTIANGIOGENIC AND ANTI-INFLAMMATORY ACTIVITIES OF STIGMASTANE DERIVATIVES, A NOVEL CLASS OF BROAD SPECTRUM ANTIVIRAL EFFECTORS

**LAURA ALCHÉ**

*Department of Biological Chemistry, School of Exact and Natural Sciences, IQUIBICEN-UBA-CONICET, University of Buenos Aires, Argentina*

Many viral infections are associated with the development of immunopathologies for which no vaccines and/or antiviral drugs are available yet. The treatment of these diseases usually includes corticosteroids which can result in reactivation of the virus, as it occurs in the case of Herpes simplex virus (HSV) infections. Particularly, HSV type 1 (HSV-1) triggers an ocular disease in humans named herpetic stromal keratitis (HSK) that can lead to vision impairment and blindness. HSK develops as a consequence of the arrival of inflammatory cells to the cornea in response to viral infection through the appearance of new vessels. While inflammatory cells are responsible for the elimination of HSV-1 from the eye, they cause an uncontrolled inflammatory response that culminates in the development of HSK.

We have demonstrated that a polyfunctionalized stigmastane derivative (22S,23S)-22,23-dihydroxystigmast-4-en-3-one (**1**) inhibits HSV-1 replication in both human corneal and conjunctival cell lines with no cytotoxicity, and reduces the signs of HSK in a mouse experimental model. On the other hand, compound **1** decreases IL-6 production in stimulated macrophages, a cytokine which is crucial for the development of neovascularization along HSK progression. Besides, RNA microarrays have revealed various overexpressed and repressed genes in compound **1** treated HSV-1 infected cells and activated macrophages, many of which are associated with innate responses and inflammatory processes. Thus, compound **1**, and others belonging to the same family of molecules, proved to combine antiviral and anti-inflammatory properties in the same chemical structure.

Since angiogenesis plays a critical role in initiating and promoting several diseases, such as HSK and cancer, we have studied the effect of compound **1** on capillary tube-like structures and on cell migration of human umbilical vein endothelial cells (HUVEC), as well as on VEGF expression, given that VEGF is a primary angiogenic factor operating in HSV-infected cornea and is considered a target to treat corneal neovascularization. Compound **1** significantly restrains the ability of HUVECs to form capillary tubes when added together with cells, although it does not cause any cytotoxic effect on the tubes already established, and it efficiently suppresses IL-6-stimulated HUVEC migration, in a concentration-dependent manner. In addition, compound **1** diminishes VEGF expression when induced by two different stimuli in macrophages.

*In vivo*, a significant decrease in the incidence and severity of corneal neovascularization during the development of HSK has been achieved by compound **1**, which explains the improvement of the signs of disease in the murine experimental model.

Additional benefits of compound **1** have been observed, since it exerts an antiangiogenic effect on the neovascular response induced by LMM3 cells in mice, by reducing the number of neovessels in a murine model of breast cancer. This novel effect of compound **1** is not shared with other compounds belonging to the same family of synthetic analogs with antiviral and anti-inflammatory properties, which lead us to conclude that the antiangiogenic effect of compound **1** is not a consequence of its anti-inflammatory activity.

In summary, the synthetic stigmastane designed as compound **1** would be a promising compound not only to cure an immunopathology of viral origin like HSK, but also to improve other diseases where angiogenesis is the major pathogenic factor, as in the case of solid tumors.

## HIGH-THROUGHPUT SEQUENCING GENOMIC APPROACHES TO UNDERSTAND KSHV/ HHV-8 ONCOGENESIS IN CELL AND MOUSE MODELS OF KAPOSI'S SARCOMA

**JULIAN NAIPAUER (1), DARIA SALYAKINA (2), MARTIN ABBA (4) LUCAS CAVALLIN (1), VYTAS DARGHIS-ROBINSON (1), SANTAS ROSARIO (1), ENRICO CAPOBIANCO (2), COURTNEY PREMER (3), JOSHUA HARE (3), PASCAL GOLDSCHMIDT-CLERMONT (1) AND ENRIQUE A. MESRI (1)**

*(1) Miami CFAR, Department and Graduate Program of Microbiology and Immunology; Viral Oncology Program, Sylvester Comprehensive Cancer Center (2) Center for Computational Science and (3) Interdisciplinary Stem Cell Institute; University of Miami Miller School of Medicine, Miami FL, USA. (4) CINIBA, Facultad de Ciencias Medicas, Universidad Nacional de La Plata, La Plata, Argentina.*

Human viral oncogenesis is the consequence of the transforming activity of virally encoded oncogenes and non-coding RNAs in combination with host oncogenic alterations. In the case of Kaposi's sarcoma (KS) and its etiologic agent, KS herpesvirus (KSHV/ HHV-8), there is a lack of experimental systems to dissect viral and

host contributions to the KS malignant phenotype. Using proteomic RTK arrays we found that PDGFRA the major oncogenic driver in our model of KSHV-driven mouse tumorigenesis and a key therapeutic target. We present two high throughput studies derived from this model. First we use RNA sequencing of KSHV Bac36 transfected mouse endothelial cells (mECK36) and their derived KSHV+ve and KSHV-ve tumors as a unique model to dissect genetic mechanisms of KSHV dependent and independent sarcomagenesis in an unbiased hi-throughput fashion since the system allows for unique experimental comparisons in the same cell and KS-like tumor types: 1) KSHV+ve vs KSHV-ve mECK36 were used to study KSHV mediated effects "in vitro" 2) KSHV+ve mECK36 grown in vitro and in tumors were used to study "in vitro" vs "in vivo" variations of host and viral gene expression induced by micro-environmental cues as well as the occurrence of host mutations in the tumors 3) KSHV+ve mECK36 vs KSHV-ve mECK36 tumors were used to dissect the role of KSHV genes and non coding RNAs in tumorigenesis by comparing mECK36 tumors driven by KSHV vs mECK36 tumors driven by host mutations. We performed Illumina, stranded, RNA seq analysis of all KSHV stages of this cell and animal model. Analysis of the host and viral transcriptome was used to characterize mechanisms of KSHV dependent and independent sarcomagenesis as well as the contribution of host mutations. Most significant results of analysis indicate that KSHV-driven in vivo growth display tumorigenesis pathways occurring predominantly by activation of developmental pathways while KSHV-ve tumors, driven by PDGFRA D842V activating mutations, occur with a predominance of proliferative pathways. Mutational analysis of mECK36 cells and tumors revealed a surprising set of mutations in inflammation/immune response related genes, absent in mECK36 cells but present in all mECK36 tumors in the same location. This indicates that these mutations should be the consequence of "in vivo" clonal selection of few mutated mECK36 cells of the population. This result suggests that in the context of in vivo tumorigenesis both these mutations and the virus may determine tumor growth. Our second model is a defined model of in vitro to in vivo tumorigenesis whereby KSHV is able to induce tumorigenesis in mouse mesenchymal stem cells (MSCs) only when infected MSCs are exposed to culture media reproducing tumor angiogenesis microenvironment conditions. Our results defines two useful cell and animal models to uncovered novel specific aspects of the interplay between host oncogenic alterations, virus-induced as well as environmentally induced transcriptional effects in the context of KSHV sarcomagenesis.

## (341) NEUTROPHIL-COXSACKIEVIRUS INTERACTION

**LEONARDO RIVADENEYRA[1], DENISE KVIATCOVSKY[2], SILVIA DE LA BARRERA[2], RICARDO MARTÍN GOMEZ[3], MIRTA SCHATTNER[1]**

*[1]Laboratorio de Trombosis Experimental e [2]Inmunología de Enfermedades Respiratorias, Instituto de Medicina Experimenta, CONICET-Academia Nacional de Medicina. Buenos Aires, Argentina. [3]Laboratorio de Virus Animales, Instituto de Biotecnología y Biología Molecular - UNLP-CONICET, La Plata, Argentina*

Coxsackieviruses B (CVBs) belong to the genus Enterovirus within the Picornaviridae family. After infection by the oral route and before viremia, CVBs replicate in lymphoid tissues, such as the tonsils and the Peyer's patches. This early interaction with immune cells has been poorly studied. In this work, we focused in the CVB-neutrophil (Neu) interaction in order to clarify the role of these inflammatory cells in the CVB infection.

For this aim, Neu were incubated with CVB and one day later viral RNA and infectious virus were searched in cells and supernatants. RT-PCR and flow cytometry (FC) studies, showed the presence of both, viral RNA and antigen in Neu-infected cell pellets. Infectivity assays confirmed the presence of infectious viral particles in the supernatants. To determine whether this Neu-CVB interaction triggered cell activation we first analyzed expression of CD11b. FC analysis showed increased levels of this adhesion cell receptor that correlated with and augmented Neu adhesion to extracellular matrix proteins such as fibrinogen and fibronectin (acid phosphatase activity).

Observation of nuclear morphological changes by fluorescence microscopy and FC showed that apoptosis was significantly lower in infected cells compared to control samples. This increase of cell survival might be due to the release of proinflammatory cytokines by Neu-infected cells. In this regard, increased levels of IL-6, IL-1$\alpha$ and TNF-$\alpha$ production were detected by ELISA in CVB-Neu supernatants. Moreover, these supernatants also showed increased chemoattractant (Boyden chamber technique) and myeloperoxidase activity (ELISA). The interaction of CVB-Neu induced low levels of neutrophil extracellular traps (NETs) that were significantly potentiated in the presence of TNF-$\alpha$. Our results indicate that interaction of Neu-CVB results in Neu activation and increased cell survival. This activation may play an important role in the subsequent pathogenesis of CVB infection.

## SAIC SYMPOSIUM XII

## ENVIRONMENTAL HEALTH

### LOSING OUR MINDS: THE ONGOING CHEMICALS' ATTACK ON OUR CHILDREN'S BRAINS

**MARICEL V. MAFFINI[1] AND THOMAS G. NELTNER[2]**

*[1]Independent Consultant, Maryland, US, [2] Environmental Defense Fund, Washington, DC, USA*

According to the WHO, non-communicable diseases underlie almost two-thirds of all global deaths, and its incidence has increased over the past 40 years, in part due to environmental chemical exposures. Developmental disabilities affect millions of people and have a great impact on their lives, their families and the societies where they live. The prevalence of disorders such as autism, attention deficit hyperactivity disorder as well as subclinical decrements in brain function cannot be explained solely as genetic diseases. Exposures to environmental chemicals, especially during prenatal and early postnatal life, are one likely explanation for some of the decrements. The current chemical risk assessment approach is typically based on the toxicity caused by a single chemical on a variety of organs without acknowledging additional exposures to other chemicals also affecting the same organ or system. We analyzed data from the US Food and Drug Administration toxicology database, high-throughput screening ToxCast and Tox21 programs, and data obtained through Freedom of Information Act and available in the public domain. We identified more than 300 chemicals allowed in food, among them were 44 food ingredients, 109 food contact substances and 86 pesticides. These chemicals could be present in any diet in any combination that may have potential harmful effects on the developing brain. Each individual chemical may or may not be harmful if it were the only one present, but we know next to nothing about their cumulative biological effects on the brain. An expanded cumulative risk assessment approach is needed, and it should focus on health outcomes, like developmental disabilities, arising from the accumulation of effects of multiple chemicals on the brain. We must move beyond treating chemical exposures as isolated incidents and look at their cumulative biological effects on organs and their role in the onset of chronic diseases. The time has come to overhaul chemical risk assessment.

### PESTICIDES AND FERTILITY: THE EFFECTS OF A BRIEF POSTNATAL EXPOSURE ON UTERINE DEVELOPMENT AND FEMALE FERTILITY

**JORGELINA VARAYOUD, MARÍA MERCEDES MILESI, PAOLA INÉS INGARAMO, MARLISE GUERRERO SCHIMPF, JORGE GUILLERMO RAMOS, MARÍA PAULA GASTIAZORO, VIRGINIA LORENZ, MÓNICA MUÑOZ-DE-TORO, ENRIQUE H. LUQUE**

*Instituto de Salud y Ambiente del Litoral (ISAL), Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral - Consejo Nacional de Investigaciones Científicas y Técnicas, Santa Fe, Argentina.*

The developmental programming hypothesis suggests that abnormal stimuli that occur during critical periods of development can permanently reprogram normal physiological responses and, consequently, give rise to reproductive health effects later in life. Early life exposures to chemicals in general, and pesticides in particular, have been associated with reproductive pathologies such as infertility and gynecologic tumors. Our research focuses on the effects of pesticides exposure on uterine development and their lasting consequences manifested later in life. The pesticides that we evaluated are the insecticide endosulfan and the herbicide glyphosate. In Argentina, glyphosate-based herbicides are the most commonly used, and although endosulfan has been banned in 2013, large quantities of this chemical continue to contaminate the environment because of its high persistence and lipophilicity. Using a rat model of early postnatal exposure we observed that low doses of endosulfan and low doses of a glyphosate-based herbicide disrupt the expression of genes that regulate uterine development and differentiation during the pre-pubertal period. In addition, we studied long-term effects on: 1) reproductive performance, 2) implantation and post-implantation processes, and 3) epigenetic modifications of endocrine-dependent genes. The results showed that both pesticides affected female fertility but in different ways. Low doses of endosulfan decreased the number of implantation sites. In the case of the glyphosate-based herbicide, there was an increased number of resorption sites. To address the effects of postnatal pesticide exposure on the pregnant uterus at the molecular level, we evaluated the endometrial proliferation and the expression of implantation and decidualization-associated genes. Both pesticides impaired endometrial proliferation and altered the expression of endocrine-regulated gene pathways. In addition, we found modifications on DNA methylation status of uterine genes,

showing evidence of epigenetic regulation of altered gene expression due to a postnatal pesticide exposure. Based on the evidence presented here and previously published data, we conclude that some pesticides are likely to diminish fertility in a laboratory animal model. More studies are needed to identify whether these or other pesticides may contribute to the decline in human fertility observed in the past decades.

## EXPOSURE TO AIR PARTICULATE MATTER: MECHANISMS UNDERLYING LUNG AND CARDIOVASCULAR EFFECTS

**PABLO EVELSON**

*Universidad de Buenos Aires. CONICET. Instituto de Bioquímica y Medicina Molecular (IBIMOL). Facultad de Farmacia y Bioquímica. Ciudad de Buenos Aires, Argentina.*

The World Health Organization reports that in 2014 3.7 million deaths were recorded as a result of air pollution exposure. This mortality has been pointed out by several epidemiological studies, which have shown a positive correlation between decreased air quality levels and adverse health effects. Air pollution is a complex heterogeous mix whose complexity is increased due to the variation in its components, between places and over time. The most important pollutants in ambient air which are of concern regarding health effects, include sulfur dioxide ($SO_2$), nitrogen oxides (NOx), carbon monoxide (CO), volatile organic compounds (VOCs), and particulate matter (PM). Of the different air pollutants, it is accepted that PM is the major concern from a health perspective. Epidemiological studies have shown that the exposure to PM, at levels experienced by populations throughout the world, contributes to pulmonary and cardiac disease through multiple mechanistic pathways that are complex and interdependent. Experimental evidence suggests a series of events that are triggered by pollution-induced pulmonary inflammatory reactions and oxidative stress with an associated risk of vascular dysfunction, altered cardiac function, and obstructive pulmonary diseases. Pulmonary inflammation is the first event observed after PM exposure. A critical component of the inflammatory response to particles in the lung is the release of cytokines from activated macrophages and lung epithelial cells, resulting in neutrophil recruitment. This response may be caused by the deposition of PM into the alveolar space in the lung, inducing the release of cytokines from alveolar macrophages. The release of proinflammatory mediators from PM-exposed macrophages is a key event that causes cytokine release from lung epithelial cells, thus amplifying the inflammatory response. The activation of inflammatory cells leads to the generation of reactive oxygen and nitrogen species. It is understood that the oxidative stress caused by the activation of the inflammatory system, plays an important role in the deleterious effects of PM in multicellular organisms. Another proposed mechanism that may lead to oxidative damage is the direct generation of ROS at the surface of the particles. This is supported by the concept that the particle surface offers a unique physicochemical interface to catalyze reactions resulting in oxidant production. The interaction of PM with membrane components was recognized by the presence of free radicals and oxidants on the particle surface. Moreover, PM can also contain a large number of soluble metals that have the ability of redox cycling. The involvement of transition metals, such as Fe, Va, Cr, Mn, Co, Ni and Cu, which are able to catalyze Fenton-type reactions and generate hydroxyl radicals, has been proposed. Once the lung inflammatory response is initiated, it develops into a systemic oxidative stress and inflammatory response, characterized by alterations in circulating factors and cells associated with inflammation and oxidative damage. It has been proposed that the release of proinflammatory and oxidative mediators can alter heart $O_2$ metabolism and cardiovascular function. Given that mitochondria play an essential role in cellular $O_2$ and energetic metabolism, several authors suggested that mitochondrial dysfunction is a key feature in the development of cardiac alterations during the exposure to air pollution PM. Taking all this into account, we evaluated cardiac $O_2$ metabolism and contractile function, focused on mitochondrial function, in a mice model of acute exposure to Residual Oil Fly Ash (ROFA), a well-known PM surrogate. Our results indicate that PM exposure decreases heart $O_2$ metabolism, probably due to a mitochondrial dysfunction. Regarding cardiac function, we observed the myocardium fails to properly sustain contractile work when work output is increased in mice exposed to PM. Interestingly, pretreatment with Infliximab, a chimeric monoclonal antibody that blocks TNF-$\alpha$ biological activity recovered the positive correlation between cardiac contractile state and $O_2$ consumption. These findings support the notion that systemic inflammation is a key pathway in the alterations in cardiac function observed after PM exposure. A better understanding of the mechanisms underlying PM induced health problems would allow a more targeted approach to face the toxic effects of PM, and could possibly provide different ways to decrease individual sensitivity to PM.

## (279) HEXACHLOROBENZENE INDUCES HYPERPLASIA AND ALTERS MOUSE MAMMARY BRANCHING MORPHOGENESIS THROUGH ARYL HYDROCARBON RECEPTOR

**NOELIA MIRET[1], EVA RICO-LEO[2], CAROLINA PONTILLO[1], ELSA ZOTTA[3], PEDRO FERNÁNDEZ SALGUERO[2], ANDREA RANDI[1]**

*[1]Universidad De Buenos Aires, Facultad de Medicina, Departamento de Bioquímica Humana, Laboratorio de Efectos Biológicos de Contaminantes Ambientales, Argentina. [2]Universidad de Extremadura, Facultad de Ciencias, Departamento de Bioquímica y Biología Molecular y Genética, Laboratorio de Biología Molecular del Cáncer, Badajoz, España. [3]Universidad de Buenos Aires, Facultad de Medicina, Departamento de Ciencias Fisiológicas, Sección Patología, Laboratorio de Fisiopatogenia, Buenos Aires, Argentina.*

Hexachlorobenzene (HCB) is an environmental pollutant that weakly binds to the aryl hydrocarbon receptor (AhR), being able to trigger AhR-dependent or -independent effects. Previous results showed a dose dependent effect of HCB in mouse mammary epithelial cells (NMuMG): 0.05 μM induced cell migration and transforminggrowth factor-β1 (TGF-β1) signaling, whereas 5 μM reduced cell migration, promoted cell cycle arrest, and stimulated AhR pathway. Our objective was to evaluate the HCB action in mammary gland from +/+AhR and -/-AhR C57BL/6N mice *in vitro* and *in vivo*. Because perturbations in mammary gland during puberty may enhance risk for later adverse effects, 4 weeks-old female mice were exposed to HCB (3 mg/kg body weight) for 21 days. Whole mount and immunohistochemistry results showed HCB to increase ductal hyperplasia (75% $p<0.05$), cell proliferation (PCNA levels, 100% $p<0.01$), nuclear estrogen receptor levels (80% $p<0.05$), branch density (15% $p<0.05$), and the number of terminal ends buds (18% $p<0.05$) in +/+AhR mammary gland. Interestingly, -/-AhR mice showed an increase in ductal hyperplasia (68% $p<0.05$) and growth (40% $p<0.05$) in absence of HCB treatment, thus revealing the importance of AhR in mammary development. However, HCB produced no changes in -/-AhR mouse mammary gland. Because interactions between epithelial cells and the stroma impacts in mammary and cancer development, immortalized mouse mammary fibroblasts (FGM) +/+AhR and -/-AhR were exposed to HCB (0.05 and 5 μM). 5 μM HCB enhanced α-smooth muscle actin expression (immunofluorescence, 508% $p<0.001$) and decreased TGF-β receptor II mRNA levels (RT-qPCR, 55% $p<0.05$) in FGM AhR+/+, resembling the phenotype of transformed cells. Accordingly, their conditioned medium was able to increase NMuMG cell motility (scratch motility assay, 84% $p<0.05$). These results show that environmental HCBconcentrations alter mammary branching morphogenesis, likely leading topreneoplastic lesions or enhanced malignancy.

## SAIC SYMPOSIUM XIII

## METABOLISM AND NUTRITION

### MODULATING BIOENERGETIC METABOLISM FOR CANCER THERAPY

**MARCELA S. VILLAVERDE**

*Unidad de Transferencia Genetica. Inst. de Oncología Ángel H. Roffo, Buenos Aires, Argentina*

Cancer cells increase glucose uptake, even in the presence of adequate oxygen levels. This phenomenon is known as the Warburg effect and suggests a dependency on glycolysis, especially in rapidly growing tumors. Thus, it becomes cancer cell metabolism an attractive area of clinical and pre-clinical therapy developments. Metformin (MET) is a biguanide, clinically known as an oral well tolerated anti-diabetic drug. Numerous recent studies show that MET decreases cancer cell viability and tumor growth in different xenograft models. Furthermore, retrospective epidemiological studies revealed a decrease in the incidence of cancer in diabetic patients treated with MET. Apparently, MET modulates cell metabolism at different cell levels since MET increases glycolysis, inhibits respiratory chain complex I and ultimately inhibits mTOR leading to growth arrest and apoptosis. However, the molecular mechanisms underlying MET antitumor effects remains unclear. On the other hand, 2-deoxyglucose (2DG) is a reversible inhibitor of hexokinase, the first and rate-limiting enzyme of glycolysis. The inhibition of glycolysis decreases the production of glycolytic intermediates, which are the precursors of nucleic acids and phospholipids. In addition, depletion of glucose-6-phosphate also decreases pentose phosphate pathway (PPP) and consequently the antioxidant defenses of cancer cells. At present, different studies explored the combination of 2DG with chemotherapy as sensitizer. Here, we will describe the potential antitumor effect and the mechanism involve in the effectiveness of modulating bioenergetic pathways by MET in combination with 2DG or 6-aminonicotinamide (6AN, PPP inhibitor) on feline mammary carcinoma and melanoma cell lines as a preclinical approach of both veterinary and human disease.

## (530) INHIBITION OF CYCLIN-DEPENDENT KINASE 4 (CDK4) ACTIVITY IN ADIPOCYTES ENHANCES THEIR THERMOGENIC PROGRAM

**ANDREA PORTALES, IGNACIO MIGUEL, ANDRÉS GIOVAMBATTISTA**

*Instituto Multidisciplinario de Biología Celular (IMBICE-CONICET), Universidad Nacional de La Plata, Argentina*

Beige adipocytes are thermogenically competent cells that develop in white adipose tissue (WAT) depots in response to b-adrenergic receptors stimulation. Two general mechanisms are accepted for beige adipocytes generation: *de novo* adipogenesis from beige precursor cells, and transdifferentiation from white adipocytes. In previous studies we found that inhibition of cyclin-dependent kinase 4 (CDK4) activity in stromal vascular fraction cells (SVF) from WAT led to an increase in beige adipocyte markers. Here we wanted to assess if the inhibition of CDK4 activity is involved in white to beige adipocyte transdifferentiation. To this aim, SVF cells from the epididimal depot of C57BL/6J mice were isolated (n=4), cultured to confluence and differentiated with an adipogenic cocktail. On day 8 post-differentiation, cells were treated or not with an inhibitor of CDK4 (Palbociclib (PAL) 1μM; CTR) for 48hs. In additional experiments, after the inhibition period, CTR and PAL cells were treated with Forskolin (FSK) 10μM for 4hs in order to activate the thermogenic program (CTR-F and PAL-F). Finally, on day 10 cells were processed for total RNA extraction and RT-qPCR quantification of thermogenic (*Ucp1*, *Prdm16*), beige (*Cd137*) and general adipogenic (adiponectin) markers. Results showed that inhibition of CDK4 in adipocytes resulted in a significant increase in *Ucp1* and *Prdm16* mRNA levels in the PAL group compared to the CTR ($p<0,05$), although *Cd137* mRNA levels remained the same. Interestingly, adiponectin was expressed at lower levels in the PAL group ($p<0,05$ vs CTR). As expected, upon FSK stimulation both, CTR-F and PAL-F groups, responded with a very large induction of *Ucp1* expression ($p<0,05$ CTR-F vs CTR, PAL-F vs PAL) while no induction of *Prdm16* was found. Our results allow us to conclude that inhibition of CDK4 would be involved in upregulation of thermogenic markers in adipocytes, suggesting that CDK4 may control the basal thermogenic state of adipocytes. PICT-2013-0930.

## (751) ACTIVATION OF NFKB SIGNALING PATHWAY IN RENAL CORTEX OF FRUCTOSE-FED RATS: EFFECTS OF DIETARY (–)-EPICATECHIN

**PAULA DENISE PRINCE[1], EZEQUIEL HID[1], JORGE E TOBLLI[2], CÉSAR G FRAGA[1,3], MÓNICA GALLEANO[1]**

*(1) Cátedra de Fisicoquímica, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina. (2) Laboratorio de Medicina Experimental, Hospital Alemán, Argentina. (3) Department of Nutrition, University of California, Davis, EE. UU.*

High fructose consumption (HFC) has been associated to deleterious metabolic conditions. In the kidney, high fructose causes alterations that contribute to the development of chronic kidney disease. Among them, inflammation is a key player of renal damage and loss of function. We evaluated the capacity of the flavanol (-)-epicatechin (EC) in attenuating the NFkB dependant inflammation induced by HFC in the kidney cortex of rats. Male Sprague-Dawley rats were fed standard diet and water (C), standard diet and fructose 10% (p/v) in the drinking water (F) and standard diet with EC (20 mg/kg BW) and the fructose solution (FE) for 8 w. No changes were observed in the expression levels of TLR-4 among the experimental groups. However, the NFkB activation signaling pathway resulted significantly increased in F group respect to C and FE groups, in terms of higher levels of activating phosphorylation in IKK$\alpha/\beta$ and p65, higher levels of phosphorylation of IkB$\alpha$ in Ser32 and a higher nuclear p65/cytosolic p65 ratio, an indicator of NFkB translocation to the nucleus (+99%*, +41%*; +113%* and +37%* F vs. C). In parallel with these results, F group showed a higher superoxide anion production (+107%* F vs. C) associated with an increased expression of NOX2 and NOX4 subunits, which were not observed in C and FE groups. EC supplementation also attenuated the increased expression of the inflammatory molecules iNOS, TNF$\alpha$ and IL-6 in kidney cortex induced by HFC. Although TLR-4 expression was not altered, a possible activation of this receptor by LPS may be possible, given that in HFC models a higher intestinal permeability is observed. Other TLR-4 independent mechanisms could be responsible for NFkB activation e.g. reticulum stress-induced IKK phosphorylation, triggered by metabolic surplus. This work supports the anti-inflammatory effect of EC, describing its modulation on NFkB signaling pathway in a context of chronic inflammation in rat kidney. *$p < 0.05$

## (845) METABOLIC SYNDROME AND NEUTROPHIL RETENTION IN THE LUNG: A MOUSE MODEL

**MARIA CECILIA DELLA VEDOVA, FLORENCIA MARTINA SOLER GARCIA, LUCIA BEATRIZ FUENTES, LUCAS DAMIAN SANTILLA, SANDRA ESTHER GOMEZ MEJIBA, NIDIA NOEMÍ GÓMEZ, DARÍO CEFERINO RAMÍREZ**

*Laboratory of Experimental and Translational Medicine, Laboratory of Molecular Biology, Laboratory of Experimental Therapeutics, Laboratory of Morpho-Physiology--IMIBIO-SL-CONICET-UNSL, San Luis, Argentina*

The metabolic syndrome (MS) is a deadly metabolic abnormality-associated to obesity. The pulmonary microvasculature is a sink of circulating neutrophils; as well as is highly sensitive to small changes in the systemic oxidative/inflammatory profile, as occur in obesity. Previously, we have characterized a MS-mouse model in which animals were fed for 16 weeks a 22% p/p chicken-fat rich diet and 10 % fructose in the drinking water. These animals show several features of MS including obesity, central obesity, insulin resistance, hypertension, dyslipidemia and streatohepatitis. Using this model we envisioned to test whether MS predispose to retention/activation of neutrophils and how it affects whole-body insulin resistance (IR). To accomplish this goal we studied MS and control mice (B6 mice fed for 16 weeks with low-fat diet/tap water). MS animals had more IR than control mice. The MS mice had also higher concentration of inflammatory mediators than control mice. The lung tissue of MS mice was lighter and expressed more inflammation mediators (TNF-$\alpha$; IL-6 and inducible nitric oxide) than the control lung. The lung of MS mice had more neutrophils (NIMP-$14^+$ cells), myeloperoxidase (MPO) activity and chlorotyrosine than control mice. ICAM-1 expression in MS mice's lung tissue was higher than control mice. In relation to control mice, intratracheal instillation (it) 2.5 ug lipopolysaccharide (LPS)/mouse to MS mice caused more retention/activation of neutrophils, ICAM-1 expression, MPO activity, chlorotyrosine, circulating inflammatory mediators and worse IR. These effects where damped by it of 5 nmol of 5,5-dimetil-1-pirrolina *N*-oxido (DMPO)/mouse. Our data suggest that retention/activation of neutrophils in the lung may be a potential therapeutic target to reduce IR and other complications of obesity. Supported by PROICO 2-3214 & PICT-2014-3369 (toDCR), PROICO 10-0414 (ToSEGM) and PIP2015-2017-112215-0100603CO (To DCR, SEA & SEGM).

## (567) EFFECTS OF HEME OXYGENASE INDUCTION ON OXIDATIVE STRESS MARKERS IN THE LIVER OF INSULIN RESISTANT RATS

**MORENA WISZNIEWSKI, CAROLINA VECINO, JUAN SALVADOR CALANNI, SILVIA SANCHEZ PUCH, CORA BEATRIZ CYMERYNG, ESTEBAN MARTÍN REPETTO**

*Laboratorio de Endocrinología Molecular (LEM). Centro de Estudios Farmacológicos y Botánicos –CEFYBO/CONICET. Facultad de Medicina, Universidad de Buenos Aires, Argentina*

Insulin resistance (IR) is a key factor involved in the pathogenesis of non alcoholic fatty liver disease along with obesity and type 2 diabetes. As heme oxygenase-1 (HO-1) has been recognized as an antioxidant enzyme playing a role in cellular defense mechanisms the aim of this study was to examine the effects of pharmacological manipulation of HO-1 on cytoprotective systems in the liver of IR rats. Male Wistar rats were randomly distributed in different groups: control (C), sucrose-rich diet (SRD, 30% sucrose in the drinking water over 12 weeks). Hemin treatment (15 mg/kg/48h, ip) was initiated after 10 weeks of diet (H and SRD+H groups) and continued for 2 weeks. Insulin sensibility was evaluated with an insulin tolerance test. Serum samples were obtained for glucose and triglyceride (TG) determination as well as liver tissue for the analysis of superoxide dismutase (SOD) and catalase (CAT) activities. Results indicate that administration of SRD (SRD and SRD+H) correlates with a decrease in insulin sensitivity. No differences in glycaemia or body weight were observed due to pharmacological or dietary treatment vs C. TG levels were increased only in SRD group vs C ($p<0.05$). HO-1 induction was determined by western blotting in liver tissues of animals under SRD and/or H treatment. In addition, we detected an increase in the activities of SOD and CAT in samples obtained from SRD-treated rats, an effect that was prevented by H treatment: 1) SOD activity (U/mg protein) mean±SEM, (n=3/group), C 5.3±0.4, H 6.7±0.9, SRD 20.3±2.9 $p<0.001$ vs C, SRD+H 9.0±1.2, $p<0.01$ vs SRD. 2) CAT activity (mM $H_2O_2$/min/mg protein) mean±SEM, (n=4/group): C 1.48±0.02, H 1.03±0.09, SRD 3.73±0.72 $p<0.01$vs C, SRD+H 1.78±0.39, $p<0.05$ vs SRD. In summary our results suggest that long term administration of SRD to rats generates oxidative stress in the liver leading to an increase in the activity of antioxidant enzymes. We also suggest that HO-1 induction by H could attenuate these effects.

## (281) ADAPTIVE RESPONSES OF CYTOSOLIC SUPEROXIDE DISMUTASE AND CATALASE IN RAT BRAIN AFTER ACUTE IRON AND COPPER OVERLOADS

**JUAN MANUEL ACOSTA[1], ROSARIO NATALIA MUSACCO SEBIO[1], CHRISTIAN MARTÍN SAPORITO MAGRIÑÁ[1], MAURICIO CASTRO PARODI[2], ALICIA DAMIANO[2,3], JULIÁN FUDA[4], HORACIO TORTI[4], NIDIA FERRAROTTI[5], ALBERTO BOVERIS[1,6], MARISA GABRIELA REPETTO[1,6]**

*Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, (1) Cátedra de Química General e Inorgánica y (2) Cátedra de Biología Celular y Molecular. (3) Consejo Nacional de Investigaciones Científicas y Técnica, Instituto de Fisiología y Biofísica Bernardo Houssay (IFIBIO-CONICET), (4) Universidad de Buenos Aires, Facultad de Farmacia y Bioquímica, (4) Cátedra de Física, (5) Departamento de Bioquímica Clínica. (6) Consejo Nacional de Investigaciones Científicas y Técnica, Instituto de Bioquímica y Medicina Molecular (IBIMOL, UBA-CONICET), Buenos Aires, Argentina*

The time course of the genomic response that involves the expression of SOD1, catalase and Nrf2 in rat brain was evaluated in the period of 0-24 h after iron (Fe) and copper (Cu) ions loads. The molecule that seems most likely to be the signal for the genomic transcription is a soluble phospholipid hydroperoxide (ROOH), where R indicates a 4-6 carbon chain, that immediately increases its steady state concentration along with the increased rates of lipid peroxidation. The hypothesis is that the activation of Nrf2-ARE signaling pathway, one of the physiologic mechanisms in cellular defense against oxidative stress that controls the expression of genes whose proteins products, is involved in detoxification and elimination of reactive oxidants and by enhancing cellular antioxidant content. The aim of the present study was to determine the characteristics of the adaptive response in rat brain after Fe and Cu ions overloads. The activities and expressions of brain cytosolic superoxide dismutase (SOD1), and catalase were determined as well as the levels of the regulatory transcription factor Nrf2 in the cytosol and nucleus. The ratio of reduced glutathione/oxidized glutathione (GSH/GSSG) was determined as a reflection of the –SH/-SS- ratio of regulatory control proteins of neuronal metabolism and function. Rats (220 ± 8 g) were given single doses of 13.5 mg $FeSO_4$/100 g or of 2.8 mg $CuSO_4$/100 g and the brain response to these overdoses was followed. SOD1, catalase, glutathione transferase and Nrf2 increased their activities/expressions in an adaptive response to oxidative stress: after 24 h of Fe and Cu administration, expression was increased: SOD1, 2.5 and 1.8 times; catalase 2 and 2.5 times ($p<0.01$). Nrf2 expression increased 1.5 times after 6 h ($p<0.01$). Conclusions: Nrf2-ARE signaling pathway, associated to GSH homeostasis, regulates brain antioxidant enzymes and ROOH seems to be the likely signaling molecule for the adaptive response after Fe and Cu loads.

## (342) MATERNAL FRUCTOSE RICH DIET INTAKE THROUGHOUT GESTATION INHIBITS THE ADIPOGENIC POTENTIAL OF WHITE ADIPOSE TISSUE PRECURSOR CELLS FROM THE MALE OFFSPRING

**ANA ALZAMENDI[1], GUILLERMINA ZUBIRÍA[1], ANDREA PORTALES[1], EDUARDO SPINEDI[2], ANDRÉS GIOVAMBATTISTA[1]**

*1. Unidad de Neuroendocrinología. Instituto Multidisciplinario de Biología Celular (CONICET La Plata-CICPBA-UNLP). La Plata. (1900). 2. Centro de Endocrinología Experimental y Aplicada (CONICET La Plata-UNLP). La Plata, Argentina.*

Previous studies from our group indicate that fructose rich diet (FRD) consumption during gestation induces a decrease in adipose precursor cells (APCs) from retroperitoneal adipose tissue (RPAT) thus reducing local adipocyte number and mass in the adult male progeny. These changes favor adipocyte hypertrophy and a distorted adipokine secretion pattern. We now evaluated the impact of FRD intake by the gestating dams on the adipogenic capacity of stromal vascular fraction (SVF) cells from RPAT, of their adult male progeny. On pregnancy day 1, dams were provided with either tap water alone (control) or containing fructose (10% w/v in drinking water; FRD) and fed *ad libitum* with chow up to delivery. Lactating dams and their pups (between 21 and 60 of age) received water and chow *ad libitum*. C and F animals will come from control and FRD dams, respectively. On experimental day (age 60 days) RPAT pads were dissected and SVF cells were isolated. The mRNA expression levels of adipogenic potential markers were assessed by qPCR in RPAT SVF cells and pads. PPARγ expression was quantified in differentiating cells and markers of terminal differentiation were also evaluated. Our data indicate that local F SVF cells express lower and higher levels ($p<0.05$) of CD34 and Pref-1, respectively. Immunofluorescence for PPARγ labeled cells (on day 4 post-differentiation) was lower in F than C animals ($p<0.05$). On day 10 post-differentiation, a lower number of differentiating F cells was noticed ($p<0.05$ vs

C). Increased Ob and LPL gene expression was found in RPAT pads from F rats ($p<0.05$). These results indicate that pre-natal nutritional intervention induces in adult male progeny a decrease in the adipogenic potential of RPAT APCs, favoring an unhealthy pad mass expansion (hypertrophic). A dysfunctional RPAT strongly contributes to develop multiple endocrine-metabolic disorders. PICT-2013-0930/CICPBA/FPREDM.

## SAI SYMPOSIUM I

## IMMUNITY AGAINST TUMORS

### CONCOMITANT TUMOR RESISTANCE: A PUTATIVE MECHANISM TO CONTROL METASTATIC GROWTH.

**RAÚL RUGGIERO, PHD.**
*IMEX-CONICET, Academia Nacional de Medicina, Buenos Aires, Argentina.*

### TUMOR INFILTRATING DYSFUNCTIONAL CD8+ T CELLS: A POTENTIAL IMMUNOMODULATORY ROLE.

**CAROLINA MONTES, PHD.**
*CIBICI-CONICET, Facultad de Ciencias Químicas,*
*Universidad Nacional de Córdoba, Argentina.*

### UNWELCOME COMPLEMENT: IMMUNOSUPPRESSIVE FUNCTIONS OF COMPLEMENT IN TUMORS AND METASTASIS-TARGETED ORGANS.

**MACIEJ MARKIEWSKI, MD, PHD.**
*Department of Immunotherapeutics and Biotechnology, Texas Tech University Health Science Center*
*School of Pharmacy, Abilene, Texas, USA.*

### (242) LOW PH IMPAIRS COMPLEMENT-DEPENDENT CYTOTOXICITY AGAINST IGG-COATED TARGET CELLS

**EZEQUIEL DANTAS, FERNANDO ERRA DIAZ, PEHUÉN PEREYRA GERBER, ANTONELA MERLOTTI, AUGUSTO VARESE, MATÍAS OSTROWSKY, JUAN SABATTÉ, JORGE GEFFNER**
*CONICET, Consejo Nacional de Investigaciones Científicas y Técnicas y UBA, Universidad de Buenos Aires.*

Extracellular acidosis is a hallmark of inflammatory conditions and solid tumors. However, few studies have addressed the influence of extracellular pH on the immune response. Here, we analyzed whether low pH could modulate complement-dependent cytotoxicity (CDC) against IgG-coated cells. Using human serum as a complement source, we found that extracellular pH values of 6.0 and 5.5 inhibit CDC against either the B cell line Raji coated with the chimeric anti-CD20 mAb rituximab (% inhibition = 48 ± 8 and 92 ± 7, respectively, n=6, $p<0.01$ vs controls) or PBMCs coated with the humanized anti-CD52 mAb alemtuzumab (% inhibition = 43 ± 6 and 88 ± 5, respectively, n=5, $p<0.01$ vs controls). Interestingly, low pH also impaired CDC assessed in the more physiologic milieu of whole blood (% inhibition at pH 6.0 and 5.5 = 49 ± 7 and 94 ± 7, respectively, n=5, $p<0.01$ vs controls). Suppression of CDC by low pH was shown to be a reversible phenomenon associated to the inhibition of both, the classical and alternative pathways of complement activation, which resulted in a reduced generation of C3a and C3b. This suggests that the major functions of the complement system triggered by IgG antibodies would be impaired by low pH. Local acidosis is a common feature of inflammatory reactions associated to infectious, allergic, vascular, autoimmune, and cancer diseases. Our observations suggest that in all these conditions, extracellular pH might exert important immunomodulatory effects by inhibiting the ability of IgG antibodies to activate the complement system.

## (414) INTRINSIC ROLE OF GALECTIN-1 IN THE CONTROL OF THE FUNCTIONAL PROPERTIES OF IMMUNE CELLS IN A PROSTATE CANCER CONTEXT

**ENRIQUE SEBASTIÁN CORAPI[1], GUSTAVO EZEQUIEL CARRIZO[1], LAURA GIRIBALDI[2], FELIPE MARTIN JAWORSKI[1], GABRIEL ADRIÁN RABINOVICH[2], DANIEL COMPAGNO[1], DIEGO JOSÉ LADERACH[1].**

*[1]Laboratorio de Glicooncología molecular y funcional, IQUIBICEN-CONICET, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina. [2]Laboratorio de Inmunopatología, IBYME-CONICET, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina.*

The identification of effective new therapies for prostate cancer (PCa) requires a better understanding of the multiple molecular interactions between tumour cells and their associated stroma.In this context, Galectin-1 (Gal-1) plays a major role in determining the properties of the prostatic carcinoma microenvironment. The aim of this study is to elucídate whether Gal-1, in additionto promote tumor neoangiogenesis and immune regulations such as induction of apoptosis on activated T cells, inefficient antigen presentation and expansion of regulatory T cells, plays an additional role as an intrinsic regulator of CD8+ T cell functional properties. To address this concern, we used an in vitro T cell polyclonal activation model combining different cell types (purified by cell sorting) in a prostate tumor microenvironment. Thus, by combination of Gal-1 deficient (Lgals1-/-) and wild type (WT) cells, we were able to assess how the endogenous Gal-1 of each cellular compartment impacts on the CD8+ T cell properties (proliferation and citotoxicity). The absence of Gal-1 in antigen presenting cells (APCs) did not significantly modify the proliferative properties of CD8+T cells. Conversely, the absence of Gal-1 in CD4+ T cells induced a 1.21 fold increase in the proliferation of CD8+T cells. However, the most significant difference in the proliferation was obtained by absence of intrinsic Gal-1 in the CD8+ T cells themselves (2.12). Taking into account Gal-1 controls T cell proliferation, we further evaluated whether Gal-1 is relevant in controlling effector function. Our results-demonstrated that upon activation, Lgals1-/- T cells have increased ability to degranulate (evaluatedas % (1.87fold) and the content of granules (2.48 fold increase) ($p < 0.05$, t test Student). Altogether, these results place Gal-1 as a potent intrinsic molecular mechanism that down-regulates the functional properties of CD8+ T cells in PCa.

## (539) TUMOR-INDUCED IL-18 PROMOTES PD-L1 EXPRESSION ON HUMAN NK CELLS

**JESSICA MARIEL SIERRA, XIMENA LUCÍA RAFFO IRAOLAGOITIA, SOL YANEL NUÑEZ, ANDREA ZIBLAT, NICOLÁS IGNACIO TORRES, FLORENCIA SECCHIARI, CAROLINA INÉS DOMAICA, NORBERTO WALTER ZWIRNER, MERCEDES BEATRIZ FUERTES**

*Laboratorio de Fisiopatología de la Inmunidad Innata. Instituto de Biología y Medicina Experimental (IBYME-CONICET).*

Natural killer (NK) cells are important mediators in the elimination of tumor and virus-infected cells, however, novel reports show a regulatory role for NK cells in different models of autoimmunity and viral infections. We have shown that NK cells from tumor bearing mice express the inhibitory molecule PD-L1 and are able to control $CD8^+$ T cell priming to tumor antigens in vivo. Moreover, in human NK cells, direct tumor recognition through NKG2D induced PD-L1 up-regulation, which was further enhanced in the presence of peripheral blood mononuclear cells (PBMCs). Therefore, the objective of this work was to elucidate the mechanisms involved in PBMC-mediated induction of PD-L1 expression on human NK cells after tumor recognition. To this end, PBMCs were cultured with K562 tumor cells and the conditioned medium (CM) obtained was used to stimulate NK cells. The CM was able to induce PD-L1 expression on NK cells ($CD3^-CD56^+$) as assessed by flow cytometry, suggesting the involvement of soluble factors. Aiming to identify these factors, PBMCs or isolated NK cells were stimulated with different doses of NK cell-activating recombinant cytokines (IL-12, IL-15 or IL-18) or cultured with K562 cells in the absence or in the presence of blocking antibodies to IL-12, IL-15 and/or IL-18. We found that IL-12 was not able to modulate PD-L1 expression. Although PD-L1 expression was induced by IL-15 on NK cells (within PBMCs, $p<0.01$ or isolated, $p<0.01$), IL-15 blockade during the co-culture of PBMCs with K562 cells did not modulate PD-L1 expression, suggesting that it is not involved in tumor-induced PD-L1 up-regulation. Finally, we found that PD-L1 expression on NK cells within PBMCs was induced by IL-18 ($p<0.0001$); moreover, IL-18 blockade in co-cultures of PBMCs with K562 cells abrogated tumor-induced PD-L1 up-regulation ($p<0.05$). Our results demonstrate that IL-18 produced by PBMCs after tumor recognition is able to up-regulate PD-L1 expression on human NK cells.

## (848) DEFICIENCY IN THE IL-17RA/IL-17 PATHWAY AFFECTS PRIMARY AND SECONDARY ANTITUMOR RESPONSES PROMOTING TUMOR GROWTH

**CONSTANZA RODRÍGUEZ, JIMENA TOSELLO BOARI, CINTIA ARAUJO FURLAN, FERNANDO PABLO CANALE, CRISTIAN GABRIEL BECCARIA, ADRIANA GRUPPI, CAROLINA LUCÍA MONTES, EVA VIRGINIA ACOSTA RODRÍGUEZ**

*Centro de Investigaciones en Bioquímica Clínica e Inmunología. CIBICI-CONICET. Departamento de Bioquímica Clínica. Facultad de Ciencias Químicas. Universidad Nacional de Córdoba.*

The role of IL-17 cytokines in cancer remains controversial as both anti- and pro-tumoral effects have been described. We and others demonstrated that IL-17 family plays a central role for the induction of NK and CD8+ T cell (CTL) responses. As these subsets are critical for host resistance to cancer, we evaluated the role of IL-17/IL-17R in modulating anti-tumor immunity and tumor progression. To this end, B6 (WT), IL-17RA KO (RKO) and IL-17A/F double KO (DKO) mice were injected with tumor cell lines that displayed progressor (B16-OVA, B16-SIY and MCA101-OVA) and regressor (MC57-SIY) growth patterns. Tumor progression and immune responses were studied at different days (d) post-injection (pi). Upon injection of B16 cells, RKO and DKO mice showed increased tumor volume and weight in comparison to WT mice ($p<0.05$, d21pi). In contrast, tumors induced by MCA101 cells had similar volumes in DKO and WT mice. Although RKO and DKO rejected MC57 tumors as efficient as WT mice, they presented higher tumor volumes between d3-8pi ($p<0.05$). Initial studies showed that MC57-bearing DKO mice presented at d12pi reduced numbers of SIY-specific CTL in draining-lymph nodes in comparison to WT controls ($p<0.05$). In addition, SIY-specific CTL from DKO mice displayed decreased frequency of cells with memory phenotype (CD62L+CD127+). As the memory CTL response developed in WT mice upon MC57-SIY cell injection is critical to protect hosts against challenge with B16-SIY tumors, we evaluated whether the SIY-specific CTL elicited in immunized RKO and DKO mice were also efficient against challenge. Of note, while all immunized WT never develop tumor or were tumor-free at d20pi of B16-SIY cells, 100% of the immunized DKO and RKO mice developed tumors and only 50% were tumor-free at d20 post-challenge. Altogether, our results indicate that the IL-17/IL-17RA pathway likely modulate primary and secondary CTL responses against tumors and may have a protective role during certain types of cancers.

# SAI SYMPOSIUM II

# IMMUNITY AGAINST INFECTIOUS AGENTS

## IMMUNOMODULATORY PROPERTIES OF THE TREMATODE FASCIOLA HEPATICA: EFFECTS ON DENDRITIC CELLS AND MACROPHAGE FUNCTIONS.

**LAURA CERVI, PHD.**

*CIBICI-CONICET. Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Argentina.*

## HIV-MEDIATED INDUCTION OF HYPOXIA INDUCIBLE FACTOR-1 ALPHA ACTIVITY IN CD4+ T CELLS MODIFIES IMMUNOMETABOLIC PHENOTYPE AND DECREASES CELL SURVIVAL.

**MATÍAS OSTROWSKI, PHD.**

*INBIRS-CONICET. Facultad de Medicina, Universidad de Buenos Aires, Argentina.*

## HANTAVIRUSES AND THEIR TARGETS, AND THEIR TARGETS´ TARGETS, AND THEIR TARGETS´ TARGETS´ TARGETS.

**JONAS KLINGSTÖM, PHD.**

*Department of Medicine, Karolinska Institute, Stockholm, Sweden.*

## (580) MTOR INHIBITION IN TRYPANOSOMA CRUZI INFECTED MACROPHAGES PRODUCES INFLAMMATORY MEDIATORS THAT REGULATE ITS SURVIVAL.

**JORGE DAVID ROJAS MÁRQUEZ, YAMILE ANA, CINTHIA STEMPIN, FABIO CERBAN**

*Departamento de Bioquímica Clínica. Centro de Investigaciones en Bioquímica Clínica e Inmunología – CONICET, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba.*

We have previously shown that *Trypanosoma cruzi* infection in macrophages (Mo) activates mTOR and its inhibition decreased parasite replication. Besides, in rapamycin (Rap) pre-treated and infected Mo we observed reduced arginase activity and expression compared to control cells. Surprisingly, we also found reduced iNOS activity and expression. Therefore, the aim of this work was to determine alternative mechanisms involved in controlling parasite replication in Rap pre-treated and infected Mo. In this context, we evaluated ROS production. We did not found differences in ROS production after 24h post infection (pi) between Rap or DMSO pre-treated and infected Mo. Also, we evaluated whether indoleamine 2,3-dioxygenase (IDO) enzyme was involved. To do that, we inhibit IDO activity by using 1MT (1-methyl-tryptophan) and we study parasite replication by immunofluorescence (IF). We did not found differences compared to control cells without 1MT and then IDO activity is either not involved in decreasing parasite replication in Rap pre-treated and infected Mo. Consequently, to study possible mediators involved in parasite killing in BMDM from different KO mice (TLR2, TLR4, IFNa-R, Caspase-1, IL-6, TNFa-R and NLRP3), parasite load was evaluated 72h pi by IF. We observed a significant increase in parasite load in Rap pre-treated and infected BMDM from IL-6-KO, TNFa-R-KO and NLRP3-KO mice compared to WT pre-treated and infected control cells for each strain. However, parasite number stands out in BMDM from NLRP3 KO ($p<0.05$). Taking into account that NLRP3 is one of the main components of the inflammasome our current studies are focused on demonstrating its activation during infection and mTOR inhibition. We found that Rap pre-treated and infected WT BMDM showed a significant increase in NLRP3 expression at 6h pi ($p<0.05$). These results would indicate that NLRP3 activation may be one of the mechanisms involved in reducing parasite replication in Rap pre-treated and infected Mo.

## (1054) SEMINAL VESICLE FLUID INCREASES THE EFFICACY OF INTRAVAGINAL HSV-2 VACCINATION

**AUGUSTO VARESE, JOSÈ ODDI, FEDERICO REMES LENICOV, MELINA GONZÁLEZ PRINZ, ANTONELA MERLOTI, JORGE GEFFNER, ANA CEBALLOS**

*Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS), Universidad de Buenos Aires -CONICET, Buenos Aires, Argentina.*

Semen induces biological actions on the female reproductive tissues directed to modulate the immune response against paternal antigens. However, the influence of seminal plasma on the immune response against sexually transmitted pathogens has not been yet evaluated. Our aim was analyze whether the seminal vesicle fluid (SVF), might compromise the induction of a protective memory response induced by HSV-2 vaccination. SVF was extracted post-mortem from 10-week-old BALB/c males. Female BALB/c mice were vaccinated IVAG with inactivated HSV-2 ($1x10^4$ pfu/15 ìl), without or with SVF (protein concentration 5 mg/ml). Thirty days later, mice were challenged IVAG wiht HSV-2 lethal dose. Mice were examined for clinical score and survival. Draining lymph nodes (DLN) and genital mucosa cells were recovered and phenotype and cytokines production was assessed by flow cytometry, ELISA, and qPCR. We observed that SVF-vaccinated mice showed 90% survival (control 10%; n=20; $p<0.001$), and minor disease progression (1.278±0.1 Mean±SEM n=20; $p<0.001$). These mice had increased IFN-ã (n=3 $p<0.05$), TNF-á (n=3 $p<0.001$), IL-17 (n=3 $p<0.05$), IL-6 (n=3 $p<0.05$) and lower IL-10 (n=3 $p<0.05$) production at the site of infection. Also, we found SVF-vaccinated mice showed a higher frequency of memory/effector like T cells ($CD44^{high}CD62L^{low}$) and central memory T cells ($CD44^{high}CD62L^{high}$) in both CD4+ (n=3 $p<0,001$) and CD8+ (n=3 $p<0,05$) T cell compartments. These changes were shown to be associated to a marked increase in the production of TNF-á and IFN-ã (Mean±SEM n=3 $p<0,001$ and $p<0,0005$, respectively). We observe that SVF significantly increased the expression of CD86 (MFI 5982 vs 3808, n=3) in vaginal DCs 48 hs post vaccination and the frequency and the total number of DCs in DLN (n= 3, $p<0,05$). In contrast with the notion that semen acts as an immunosuppressive agent, our results suggest that SVF might induce an adjuvant effect on the female immune response against sexually transmitted pathogens.

## (2008) CONTRIBUTION OF INFLAMMASOMES TO THE CONTROL OF THE RESPIRATORY INFECTION BY BRUCELLA SPP.

**ANDREA GISELLE FERNANDEZ[1], MARÍA SOLEDAD HIELPOS[1], JULIANA FALIVENE[1], MARIANA C. FERRERO[1], SERGIO COSTA OLIVEIRA[2], PABLO C. BALDI[1]**

*[1]IDEHU (CONICET-UBA), Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. [2]Departamento de Bioquímica e Inmunología, Universidad Federal de Minas Gerais, Belo Horizonte, Brasil.*

Inhalation of infected aerosols is one of the most frequent routes for acquiring *Brucella* spp. infection. The role of IL-1β and inflammasome activation in the initial control of this infection has not been studied. To determine whether pulmonary IL-1β contributes to the control of airborne *B. abortus* infection, IL-1 receptor *knock-out* mice (IL-1R KO) and wild type (WT) controls (C57BL/6 mice) were infected by the intra-tracheal route, and lung CFU were measured at 2 and 7 days p.i. While no significant difference was found at 2 days p.i., CFU counts were significantly higher in IL-1R KO mice at 7 days p.i. as compared to WT (mean, $3.15 \times 10^7$ vs. $1.12 \times 10^6$ CFU; $p<0.01$). Pulmonary levels of KC (neutrophils chemoattractant) were significantly lower in IL-1R KO mice at 7 days p.i. (88.3 vs. 146.2 pg/ml, $p<0.05$). To determine whether inflammasomes contribute to the control of airborne *B. abortus* infection, KO mice for caspase-1 (Casp-1), NRLP3 or AIM2 were infected by the intra-tracheal route, and lung CFU and cytokines were measured at different p.i. times. CFU counts were higher in Casp-1, NLRP3 and AIM2 KO mice ($8.01 \times 10^6$, $1.18 \times 10^7$, and $5.43 \times 10^6$ CFU/ml, respectively) as compared to WT ($0.81 \times 10^6$ CFU/ml), although differences only reached statistical significance for NLRP3 ($p<0.01$). In addition, at 2 days p.i. levels of IL-1β were lower in Casp-1 KO mice than in WT (213 vs. 465 pg/ml, $p<0.01$), and the same was true for KC (96 vs. 157 pg/ml, $p<0.01$), IL-12 (73 vs. 295 pg/ml, $p<0.05$) and TNF-β (130 vs. 260 pg/ml, $p<0.001$). To determine the role of different lung cells in these responses, alveolar macrophages and pneumocytes were obtained from WT mice and were infected in vitro in the presence or absence of a Casp-1 inhibitor (Z-WEHD-FMK). For both cell types, IL-1β levels were significantly lower in the presence of the inhibitor. These results show that inflammasomes play an important role in the initial control of *Brucella* infection acquired through the airways.

## (54) B. ABORTUS MODULATES OSTEOBLAST FUNCTION THROUGH THE INDUCTION OF AUTOPHAGY

**AYELÉN IVANA PESCE VIGLIETTI, PAULA CONSTANZA ARRIOLA BENITEZ, GUILLERMO HERNÁN GIAMBARTOLOMEI, MARÍA VICTORIA DELPINO**

*Instituto de Inmunología, Genética y Metabolismo (CONICET-UBA), Buenos Aires, Argentina*

Osteoarticular brucellosis is the most common localization of human active disease. Osteoblasts are specialized mesenchyme-derived cells involved in bone formation and are considered as professional mineralizing cells. We demonstrated that *B. abortus* infection modified osteoblast metabolism by the inhibition of the deposition of organic and mineral matrix, leading to bone loss. *B. abortus* replicative vacuole involved autophagy pathway activation and on the other hand, autophagy has been involved in osteoblast metabolism. Then experiments were conducted to determine if *B. abortus* modulates osteoblast function through the activation of autophagy. To this end, *B. abortus* infected osteoblasts cells (MC3T3 E1 cell line) were lysed to determine LC3II, beclin-1 and p62 expression by Western blotting. MMPs production was determined by gelatin zymography, collagen deposition by Sirius red staining and alizarin red S staining to determine calcium deposition. *B. abortus* infection increased the levels of LC3II ($p<0.01$) and beclin-1($p<0.01$) and inhibited p62 expression indicating autophagy pathway induction. In addition, when *B. abortus* infection experiments were performed in the presence of bafilomycin A1 or chloroquine we did not observe inhibition of deposition of mineral and organic matrix ($p>0.05$). Taking together our results indicated that *B. abortus* induced the activation of autophagy pathway in osteoblast cells and this activation is involved in the modulation of osteoblast function and bone formation.

## SAI SYMPOSIUM III

## MEMORY T CELLS

### T CELL PRIMING AND MEMORY T CELL RESPONSE TO PERIPHERAL VIRUS INFECTION

**SCOTT MUELLER, PHD.**

*Dept. Microbiology & Immunology, The University of Melbourne and The Peter Doherty Institute for Infection and Immunity, Melbourne, Australia.*

### T CELLS AS SENTINELS OF THE INTESTINAL MUCOSA.

**PABLO ROMAGNOLI, PHD**

*Instituto Universitario de Ciencias Biomédicas de Córdoba (IUCBC), Córdoba, Argentina.*

### (861) ARYL HYDROCARBON RECEPTOR SIGNALING MODULATES CD8 + T CELL EFFECTOR AND MEMORY SUBPOPULATIONS DURING THE INFECTION WITH TRYPANOSOMA CRUZI.

**CONSTANZA INSFRÁN, LAURA FERNANDA AMBROSIO, XIMENA VOLPINI, HORACIO MARCELO SERRA, CLAUDIA CRISTINA MOTRÁN**

*Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI)-CONICET-UNC, Córdoba, Argentina*

The acquisition of memory T cells is defined by the generation and persistence of T cells that can provide long-lasting protection against pathogens. Signals given by dendritic cells (DC) as TCR engagement, costimulation and cytokines, co-participate in the induction of memory T cells. Thus, changes in any of the factors controlling the activation of T cells during antigen presentation by DC can regulate T effector and memory cell differentiation. Different observations suggest that AhR, is a ligand-activated transcription factor that plays important roles in several biological processes, modulates critical events in the activation of naive T cells that could modify the development of effector and memory T cells. To investigate the role of AhR activated by natural ligands generated during *T. cruzi* infection, B6 (WT) and B6 mice carrying a mutant AhR protein with reduced affinity for its ligands (AhRd) were infected with 100.000 *T. cruzi* trypomastigotes and different splenic subpopulations of CD8+ T cells were studied by FACS at day 10 and 170 postinfection (pi). AhRd mice showed significantly lower frecuency and number of CD8+ T cells specific for the inmunodominant epitope TSKB20 (ANYKFTLV) than WT mice at day 10 pi, ($p$<0,05). Interestingly, analysis of CD44 and CD127 expression that distinguish memory precursors effector cells (MPECs: $CD44^{hi}$ $CD127^{hi}$) from short lived effector cells (SLECs: $CD127^{lo}$ $CD44^{hi}$) in subpopulations of CD8+ and CD8+TSKB20+ T cells revealed that, at day 170 pi, the frequency and number of splenic MPECS was higher in AhRd than in WT mice, ($p$<0,01). The study of memory subpopulations, $CD44^{hi}$ $CD127^{hi}$ $CD62L^{hi}$ (central memory, CM) or $CD62L^{lo}$ (effector memory, EM) at day 170 pi showed that the frequency and number of splenic EM and CM CD8+TSKB20+ T cells were higher in AhRd than WT mice, ($p$<0,01). Thus, during *T. cruzi* infection AhR signaling restricts the differentiation of CD8+ memory T cells.

### (92) ALTERED EXPRESSION OF LTCD4 AND LTCD8 NAIVE AND MEMORY SUBSETS IN HIV INFECTED CHILDREN.

**ALEJANDRA URIOSTE, ESTEFANIA CAPECCE, MARCELA CANDI, JEANETTE BALBARYSKI, GRACIELA BARBONI, EDUARDO GADDI**

*División Inmunología, Hospital General de Niños Dr. Pedro de Elizalde, Ciudad Autónoma de Buenos Aires, Argentina*

CD28 and CD95 expression over LTCD4 and CD8 cells, define naïve (N) and memory (M) subsets. CD28 is a costimulatory T-cell molecule and its specific loss is associated with immune senescence. CD95 antigen promotes apoptosis pathways, and is expressed also, over activated T cells. HIV infection is characterized by LTCD4 depletion, chronic immune activation and quantitative and phenotypic changes in T cells subsets. Our aim was to describe T cells subsets levels in a cohort of HIV-infected children. The group evaluated comprised 47 HIV-infected

children, (age: 2-14 years), vertical transmission was confirmed in all patients, they were under antiretroviral therapy. LTCD4 and CD8 N (CD28+CD95-), central memory (CM) (CD28+CD95+), effector memory (EM) (CD28-CD95+), and senescence LTCD8 CD57+ subsets, were studied by flow cytometry. Clinical and virological status of all patients was also evaluated. Control samples (Co) were obtained from 10 HIV-seronegative healthy children. LTCD4 N subset percentage levels were decreased significantly ($p<0.05$) in patients with mild or severe immunosuppression: Group A (LTCD4<25%, n: 20), in comparison with children with no evidence of immunosuppression: Group B (LTCD4 ≥ 25%, n: 27), and Co, (A: 33.5±16.6 vs B: 48.5±20.6 vs Co: 67.4±9.5). Similar results were obtained with LTCD8 N (A: 10.6±10.3 vs B: 23.4±17.3 vs Co: 52.8±11.2). A significant increase in LTCD4 CM between groups A, B and Co, was also recorded (A: 58.9±17.2 vs B: 47.4±20.5 vs Co: 31.0±7.5). LTCD8 CM showed increase between A and B groups against Co (A: 24.7±10.8 vs Co: 16.0±7.1, B: 36.3±19.1 vs Co: 16.0±7.1). LTCD8 EM were increased significantly between A, B and Co (A: 57.4±20.3 vs B: 33.8±18.1 vs Co: 6.9±3.1). A positive correlation between LTCD8 EM and LTCD8 CD57+ was observed (r: 0.516, p: 0.003). Differential involvement of N subset and senescence characteristic of cytotoxic cells would be associated with persistent viral replication and repeated immune activation.

## (904) PATIENTS WITH IL-17R DEFICIENCY SHOW ALTERATIONS IN THE NK AND CD8+ T CELL COMPARTMENTS.

**JIMENA TOSELLO BOARI[1], NICOLÁS NUÑEZ[2], MIGAUD MELANIE[3], MARÍA CECILIA RAMELLO[1], CAROLINA MONTES[1], ELIANE PIAGGIO[2], ANNE PUEL[3], EVA ACOSTA RODRIGUEZ[1]**

*[1]Departamento de Bioquímica Clínica. Facultad de Ciencias Químicas. Universidad Nacional de Córdoba. Centro de Investigaciones en Bioquímica Clínica e Inmunología. CIBICI-CONICET. Córdoba – Argentina. [2]INSTITUT CURIE Laboratoire de Transfert INSERM U932. Paris - FRANCE [3]Génétique Humaine des Maladies Infectieuses. INSERM UMR 1163. Université Paris Descartes-Sorbonne Paris Cité. Institut Imagine. Paris – FRANCE.*

The IL-17 cytokine family plays pivotal roles in inflammatory diseases and in host defense against several pathogens in mice and humans. Previously, we and others have demonstrated that IL-17RA deficient mice show compromised maintenance and activation of the main cytotoxic populations (i.e. NK and CD8+ T cells (CTL)) during infections. Here, we aimed at evaluating if IL17RA deficiency affects functional cytotoxic response also in humans. To do this, we studied by flow cytometry the frequency and phenotype of NK and CTL present on peripheral blood from three patients presenting genetic mutations that prevents IL-17RA expression. Blood from healthy donors (HD) were studied for comparison. Of note, IL-17RA deficient (RA-/-) patients showed a significant lower frequency of NK cells than HD ($p=0.0004$). Moreover, CTL from these patients showed a conserved frequency but a distinctive phenotype in comparison to those from HD. Hence, total and CD45RA- CTL from RA-/- patients showed a consistently higher frequency of OX40+ ($p=0.0469$ and $p=0.0281$) and CD38+ ($p=0.0418$ and $p=0.0123$) cells and, only within CD45RA- CTL, an increased % of ICOS+ cells ($p=0.0014$). In contrast, other activation markers such as CD25 ($p=0.0061$ and $p<0.0001$) and CD69 ($p=0.008$ and $p=0.0024$) showed reduced expression while HLA-DR and 4-1BB showed no changes in expression on total and CD45- CTL from RA-/-patients. Interestingly, CTL with a phenotype of central memory (CD45RA-CCR7+), which display a high expression of IL-17RA in HD, were significantly diminished in RA-/- patients ($p=0.0070$). This reduction in the frequency of the memory subset correlated with a significant increase in the percentage of CTL with phenotype compatible with senescence (KLRG1+CD57+; $p=0.0377$) and (CD27-CD28-CD45RA-, $p=0.0205$). These results suggest that, as it has been described in mice, IL-17RA signaling may modulate the maintenance, activation and differentiation of human cytotoxic immune populations.

# SAI SYMPOSIUM IV

# PLASTICITY OF MYELOID CELLS

## DIVERSE ROLES OF MACROPHAGES IN THE INITIATION AND THE RESOLUTION OF INFLAMMATION.

**PROFESSOR DAVID M. MOSSER, PHD.**

*Cell Biology and Molecular Genetics, Maryland Pathogen Research Institute, Maryland, USA.*

## HUMAN INFLAMMATORY DENDRITIC CELLS: ONTOGENY AND FUNCTION.

**ELODIE SEGURA, PHD.**

*"Dendritic cells and antigen presentation" lab, INSERM U932, Institut Curie, Paris, France.*

## (715) M2 MACROPHAGES INHIBIT IFN-G PRODUCTION OF NK CELLS THROUGH TGF-B AND NK CELL-MEDIATED CYTOTOXICITY THROUGH CELL-TO-CELL CONTACT

**SOL YANEL NUÑEZ, ANDREA ZIBLAT, FLORENCIA SECCHIARI, NICOLÁS IGNACIO TORRES, JESSICA MARIEL SIERRA, ROMINA ELIZABETH ARAYA, XIMENA LUCÍA RAFFO, CAROLINA INÉS DOMAICA, MERCEDES BEATRIZ FUERTES, NORBERTO WALTER ZWIRNER**

*[1]Laboratorio de Fisiopatología de la Inmunidad Innata. Instituto de Biología y Medicina Experimental (IBYME-CONICET).*

Macrophages are highly plastic cells that can modify their functional response according to the surrounding microenvironment, becoming pro-inflammatory (M1) or anti-inflammatory (M2) macrophages. The outcome of the crosstalk between M1 and NK cells is well established playing a critical role in the protection against infections and tumor growth. However, the effect of M2 on NK cells is less clear. We previously demonstrated that M2 macrophages inhibit NK cell degranulation ($n$=8, $p$<0,001) and cytotoxicity ($n$=6, $p$<0,0001) against tumor cells and also IFN-$\gamma$ secretion upon stimulation with cytokines (assessed by flow cytometry and ELISA, $n$=6, $p$<0,001) compared to M1. Thus, the aim of this work was to investigate the underlying mechanisms involved in this inhibition. Accordingly, we used co-cultures of human NK cells with M1 or M2 *in vitro* polarized macrophages. Human monocytes were differentiated to unpolarized macrophages (M0) with M-CSF for 6 days and then exposed overnight to LPS and IFN-$\gamma$ or IL-4 to obtain M1 and M2 respectively. Then isolated NK cells (resting or stimulated with IL-12, IL-15 and IL-18) were co-cultured overnight with M1 or M2 cells. Transwell experiments and NK cell culture with M1 or M2 conditioned media demonstrated that the inhibition of IFN-$\gamma$ secretion was due to soluble factors while inhibition of degranulation required contact between the respective cell types. Blockade of the immunosuppressive cytokine TGF-$\beta$ in M2 macrophage-conditioned media restored the IFN-g secretion by NK cells ($n$>6, $p$<0,001) but blockade of TGF-$\beta$ during co-cultures had no effect on NK cell degranulation ($n$=4, $p$>0,05), indicating that TGF-$\beta$ is involved in silencing IFN-$\gamma$ secretion but not NK cell-mediated cytotoxicity. Therefore, we conclude that M2 negatively regulate NK cell IFN-$\gamma$ production through secretion of TGF-$\beta$ but negatively regulate NK cell-mediated cytotoxicity through the interaction between cognate receptor-ligands expressed by these cells.

## (326) FEVER-RANGE HYPERTHERMIA IMPROVES THE ANTI-APOPTOTIC EFFECT INDUCED BY LOW PH ON HUMAN NEUTROPHILS PROMOTING A PROANGIOGENIC PROFILE

**FERNANDO ERRA DÍAZ[1], EZEQUIEL DANTAS[1], CABRERA MAIA[2], JOSEFINA CASTRO MAZZA[1], CONSTANZA ARRIOLA BENÍTEZ[3], MARÍA VICTORIA DELPINO[3], NORBERTO SANJUAN[4], ANALÍA SILVINA TREVANI[5], JORGE GEFFNER[1]**

*[1]Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (INBIRS), CONICET, Facultad de Medicina, Universidad de Buenos Aires, Argentina. [2]Instituto de Investigaciones Farmacológicas (ININFA), CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina. [3]Instituto de Inmunología, Genética y Metabolismo (INIGEM), CONICET, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Argentina. [4]Instituto de Investigaciones en Microbiología y Parasitología Médica (IMPAM), CONICET, Facultad de Medicina, Universidad de Buenos Aires, Argentina. [5]Instituto de Medicina Experimental (IMEX), CONICET, Academia Nacional de Medicina, Buenos Aires, Argentina.*

Neutrophils have the shortest lifespan among leukocytes and usually die via apoptosis. We previously reported that low pH, a hallmark of inflammatory processes and solid tumors, moderately delays neutrophil apoptosis. Here we analyzed whether fever-range hyperthermia (39.5°C) was able to modulate the anti-apoptotic effect induced by low pH (pH 6.0). Hyperthermia markedly increased neutrophil survival induced by low pH (anexinV/PI staining/flow cytometry): % apoptosis = 72±5, 45±6, 70±7, and 19±3, for neutrophils cultured for 18 h at pH 7.3/37ºC, pH 6.0/37°C, pH 7.3/39.5ºC, and pH 6.0/39.5°C, respectively (p<0.001 for pH 6.0/39.5ºC vs pH 7.3/37°C, n=9). Similar results were observed when apoptosis was evaluated by different methodologies. Analysis of the mechanisms underlying this anti-apoptotic response revealed that hyperthermia further decreases cytosolic pH induced by extracellular acidosis, evaluated with BCECF-AM/flow cytometry (p<0.01, pH 6.0/37°C vs pH 6.0/39.5°C). The fact that two Na+/H+ exchanger inhibitors, EIPA(10mM) and amiloride (1mM) reproduced the effects

induced by hyperthermia suggested that it prolongs neutrophil survival by inhibiting the Na+/H+ anti-porter. Interestingly, we found that the anti-apoptotic effect induced by low pH and hyperthermia is associated to the induction of a functional profile characterized by a low phagocytic activity evaluated by fluorescence microscopy using FITC-labeled *Candida albicans* (% inhibition 56 ± 8, n=5, pH 6.0/39.5°C vs pH 7.3/37°C, p<0.001), an impairment in ROS production evaluated by DHR oxidation and flow cytometry (% inhibition >90%, n=7, pH 6.0/39.5°C vs pH 7.3/37°C, p<0.001), and a higher ability to produce the angiogenic factors VEGF, IL-8 and the matrix metallopeptidase 9 (MMP-9) evaluated by ELISA and zymography (p<0.01, pH 6.0/39.5°C vs pH 7.3/37°C, n=6). These results suggest that acting together fever and local acidosis might drive the differentiation of neutrophils into a proangiogenic profile.

## (781) BORDETELLA PERTUSSIS EFFECT ON MACROPHAGE PHENOTYPE DURING THE INFECTION.

**HUGO ALBERTO VALDEZ[1], LUCIANA BALBOA[2], JUAN PABLO GORGOJO[1], HILARIO CAFIERO[1], MARÍA DEL CARMEN SASIAIN[2], MARÍA EUGENIA RODRIGUEZ[1]**

*[1]CINDEFI (UNLP CONICET La Plata), Facultad de Ciencias Exactas, Universidad Nacional de La Plata, La Plata, Argentina. [2]Laboratorio de Inmunología de Enfermedades Respiratorias, Instituto de Medicina Experimental (IMEX)-CONICET, Academia Nacional de Medicina, Buenos Aires, Argentina.*

After bacterial products recognition macrophages initiate an immune response for removal of the microbes. Proper polarization of macrophages is critical for bacterial clearance. During microbial infection, macrophages are polarized to classically or alternatively activated cells (M1 or M2, respectively) in response to microbial components and host immune mediators. Recently we have demonstrated that *B. pertussis* (Bp) has the ability to manipulate the host defense response eventually enabling its own survival and replication inside the macrophages. In the present study we examined the evolution of macrophage phenotype during the Bp intracellular survival and proliferation. To this end, primary peripheral blood mononuclear cells (PBMC) were differentiated into macrophages in the presence of GM-CSF; GM-CSF + INF-gamma/LPS; M-CSF + IL-4 or M-CSF + IL10 to obtain the phenotypes M0, M1, M2a or M2c, respectively. Intracellular Bp infection evolution was determined by FISH staining and confocal microscopy. Macrophage expression of CD40, CD80, CD206, CD209 and CD163 was determined at 3 and 48 hours after infection. Three hours post infection macrophages cultured under all conditions displayed an increase in CD40 and CD80 expression and a decrease in CD206, CD209 and CD163 expressions as compared with the uninfected control. Forty eight hours post infection cells in which Bp infection had developed showed a decrease in CD40 and CD80 expression and an increase of CD209 and CD163 in all macrophage phenotypes (M0, M1, M2a and M2c) as compared with the uninfected population of the respective phenotype. These results indicate that early after bacterial phagocytosis macrophages develop an M1 like phenotype. However, as the intracellular infection progresses infected cells of any macrophage phenotype turned into an M2 like type, indicating the extraordinary ability of this pathogen to induce a permissive environment for its survival within human macrophages.

## (494) PDL1 SIGNALING INHIBITS MACROPHAGE'S SUSCEPTIBILITY TO M. TUBERCULOSIS-SPECIFIC CD8+ T CELL INDUCED APOPTOSIS

**GUADALUPE VERÓNICA SUÁREZ, MARÍA BELÉN VECCHIONE, FLORENCIA QUIROGA**

*Universidad de Buenos Aires. Consejo Nacional de Investigaciones Científicas y Técnicas. Instituto de Investigaciones Biomédicas en Retrovirus y Sida (INBIRS). Facultad de Medicina. Buenos Aires, Argentina.*

CD8+ T cells contribute to the optimal control of Tuberculosis infection by inducing apoptosis on infected macrophages. M. tuberculosis (Mtb) infection is associated to alternative activation of macrophages. We aimed to study the effect of macrophage activation on Mtb-specific CD8+ T cell induced apoptosis. Monocyte Derived Macrophages (MDMs) from healthy donors were activated by IFNγ or IL4 addition during 48 h and loaded with heat-killed Mtb or CEF peptide pool. Macrophages' phenotype was determined by HLA-DR, CD86, DC-SIGN and CD14 staining and flow cytometry. Then, MDMs were co-cultured with antigen-stimulated, autologous CD8+ T cells. Specific killing was assessed by 7-AAD staining. Data was analyzed by ANOVA followed by Bonferroni posttest. IFNγ activation of MDMs tended to decrease CD8-induced Mtb-specific apoptosis but it remained sig-

nificant at a ratio of 1:1 ($p<0.01$). Contrary, IL4 activation completely abrogated Mtb-loaded MDMs apoptosis. Thus, IL4 significantly reduced Mtb-specific killing compared to non-activated MDMs ($p<0.05$). IFN$\gamma$ production by CD8+ cells determined by ELISPOT was not affected by MDMs activation with IFN$\gamma$ or IL4 ($p=0.49$ by Friedman test). HLA-ABC, CD80 and CD86 expression were increased by IFN$\gamma$ or Mtb ($p<0.05$) but were unaffected by IL4 activation, suggesting that the observed differences in killing were not due to differences in antigen presentation or positive co-stimulation by macrophages. Interestingly, PDL1, but not PDL2 expression was acutely increased by IFN$\gamma$ or Mtb ($p<0.001$) and to a lesser extent, by IL4 ($p<0.05$). Also, PDL1, but not PDL2-blockade improved CD8+ T cell-mediated cytotoxicity to IFN$\gamma$ but not IL4-activated MDMs. Our results indicate that the increment of PDL1 by IFN$\gamma$ activation inhibits macrophages susceptibility to CD8+ T cell induced apoptosis.

## SAI SYMPOSIUM V

## PHYSIOPATHOLOGY AND REGULATORY MECHANISMS OF THE IMMUNE RESPONSE

### MECHANISMS OF ROBUST TRANSPLANTATION TOLERANCE.

**MARÍA LUISA ALEGRE, MD, PHD**

*Gwen Knapp Center for Lupus and Immunology Research, University of Chicago, Chicago, Illinois, USA.*

### METABOLIC PATHWAYS SHAPING IMMUNE CELL FUNCTION

**LUCIANA BEROD, PHD.**

*Institute of Infection Immunology, Twincore, Centre for Experimental and Clinical Infection, Research, Hannover School of Medicine, Hannover,Germany.*

### (484) IMPACT OF THE MACROPHAGE ACTIVATION STATE ON THE ACCUMULATION OF LIPID BODIES INDUCED BY THE TUBERCULOUS PLEURISY MILIEU

**MELANIE GENOULA[1], DENISE KVIATCOVSKY[1], AYELEN MILILLO[2], BELÉN IMPERIALE[1], EDUARDO MORAÑA[3], PABLO GONZÁLEZ-MONTANER[3], DULCE MATA-ESPINOZA[4], ERIKA GONZÁLEZ-DOMÍNGUEZ[5], CARMEN SÁNCHEZ-TORRES[5], ROGELIO HERNÁNDEZ-PANDO[4], PAULA BARRIONUEVO[2], MARÍA DEL CARMEN SASIAIN[1], LUCIANA BALBOA[1]**

*[1]Laboratorio de Inmunología de Enfermedades Respiratorias, Instituto de Medicina Experimental (IMEX)-CONICET, Academia Nacional de Medicina, Buenos Aires, Argentina. [2]Laboratorio de Fisiología de los Procesos Inflamatorios, Instituto de Medicina Experimental (IMEX)-CONICET, Academia Nacional de Medicina, Buenos Aires, Argentina. [3]Instituto Prof. Dr. Raúl Vaccarezza, Hospital de Infecciosas Dr. F.J. Muñiz, Buenos Aires, Argentina. [4]Departamento de Patología Experimental, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico. [5]Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Mexico City, Mexico.*

During intracellular bacterial infection, the eukaryotic cells show metabolic adaptations that help them to eliminate the pathogen and conversely, the pathogen tries to profit from host metabolites. The ability of *Mycobacterium tuberculosis* (*Mtb*) to persist relies on its numerous immune evasion strategies such as the dysregulation of the lipid metabolism that can lead to foamy macrophage (FM) differentiation. So far, the specific host factors leading to FM induction are unknown. We aimed to characterize whether different profiles of macrophage (M0, M1, M2a, and M2c) differ in their propensity to accumulate lipid bodies (LB) upon exposure to tuberculous pleural effusions (TB-PE). For that, LB accumulation was evaluated by oil red staining, cytokines by ELISA, pSTAT-3 and ACAT by western blot, cholesterol by enzymatic assays, and bacillary loads by colony-forming units assay. TB-PE induced a FM phenotype in M0, M2c and M1 but not in M2a (n=8). After depleting IL-10, IL-4, IFN-g, IL-1b, IL-6, or TNF-a from TB-PE, only IL-10 depletion prevented FM differentiation (n=8). TB-PE increased the levels of

intracellular cholesterol, while IL-10 depletion reduced it. Besides, PE-TB or IL-10 addition induced CD36 expression, which mediates lipids uptake, and also pSTAT-3 levels, which governs the M2c program (n=5); moreover, pSTAT-3 inhibition prevented LB accumulation (n=4). Interestingly, the expression of ACAT, the enzyme that synthesizes cholesteryl esters, was induced by TB-PE in any macrophage profile except for M2a. Finally, TB-PE promoted the intracellular growth of *Mtb* in macrophages in an IL-10 dependent manner but not in M2a (n=10). Therefore, macrophage profiles differ in their propensity to accumulate LB, process that requires the activation of the IL-10/STAT-3 axis and favours *Mtb* replication. M2a was refractory to FM differentiation lacking ACAT expression. These results contribute to our understanding of the host metabolic alterations driven by *Mtb*.

## (624) CORNEAL INJURY IN ONE EYE DISRUPTS MUCOSAL IMMUNE TOLERANCE OF THE FELLOW OCULAR SURFACE BY INDUCING SUBSTANCE P-MEDIATED NEUROGENIC INFLAMMATION.

**MAURICIO GUZMÁN, IRENE KEITELMAN, FLORENCIA SABBIONE, ANALÍA TREVANI[1], MIRTA GIORDANO, JEREMÍAS GALLETTI**

*[1]Laboratorio de Inmunología Oncológica. Instituto de Medicina Experimental. Academia Nacional de Medicina/CONICET. Buenos Aires. Argentina. [2]Universidad de Buenos Aires. Facultad de Medicina. Departamento de Microbiología e Inmunología. Buenos Aires. Argentina.*

In the clinic, it is commonly accepted that both eyes are functionally independent, but we unexpectedly observed immune tolerance perturbations in the opposite eye after unilateral manipulation in mice. Since there is no ocular lymphatic cross drainage that could account for this contralateral change, we studied the effect of a unilateral corneal lesion on conjunctival mucosal tolerance of the fellow eye. Using a delayed-type hypersensitivity (DTH) assay after s.c. immunization with ovalbumin (OVA)+adjuvant in Balb/c mice, we observed observed reduced responses in OVA-instilled mice compared with control mice ($p<0.05$). However, OVA-instilled mice in the same or in the opposite eye to the corneal burn developed full DTH responses. Consistently, mice injected with allogeneic B16 tumor cells in the subconjunctival space of the eye opposite to the corneal burn had a higher rejection rate than uninjured mice (89% vs 56%, $p<0.05$). In mice with a unilateral corneal burn, we observed an increase in the fraction of activated (CD69+ and CD25+) and interferon ã-secreting T cells and in the number of dendritic cells (CD11c+ MHC II+) in the opposite eye-draining lymph node that peaked 48-72 h after the injury ($p<0.05$). We then explored the contribution of neurogenic inflammation by using capsaicin (TRPV1 receptor agonist) and aprepitant (substance P receptor antagonist). Remarkably, unilateral ocular instillation of capsaicin also abrogated contralateral tolerance to OVA ($p<0.05$), and corneal burn-induced disruption of contralateral tolerance to OVA was prevented by aprepitant instillation ($p<0.05$). In summary, our results show that ocular mucosal tolerance was disrupted in the fellow eye after a unilateral corneal burn, and that this effect appears to be mediated by substance P-induced neurogenic inflammation. These findings could have major implications in the understanding and management of contralateral disease after single eye interventions.

## (699) PARTICIPATION OF TYPE I-II IFNS IN THE REGULATION OF THE CNS IMMUNE SURVEILLANCE AFTER SYSTEMIC INFLAMMATION.

**JAVIER MARÍA PERALTA RAMOS, CLAUDIO BUSSI, DANIELA SOLEDAD ARROYO, PABLO IRIBARREN**

*Center of Investigation in Clinical Biochemistry and Immunology (CIBICI-CONICET), Department of Clinical Biochemistry, National University of Córdoba.*

Brain-resident microglia (Mi) and peripheral recruited leukocytes, play essential roles in shaping the immune response in the central nervous system (CNS). These cells activate and migrate in response to chemokines produced during active immune responses and may contribute to the progression of neuroinflammation. Recent findings have revealed distinct roles for type I ($\alpha$ and $\beta$)-II ($\gamma$) interferons (IFNs) in the recruitment of immune cells to the CNS and highlighted the importance of this process for brain protection/repair. In this study, we assessed the participation of type I-II IFNs in the innate immune response displayed by tissue-resident microglia and

recruited inflammatory leukocytes, to better understand the contribution of these cytokines in the establishment and development of a neuroinflammatory process induced by systemic TLR4 stimulation. We characterized the molecular and cellular players involved in neuroinflammation induced by i.p. administration of lipopolysaccharide (LPS - 1.6 mg/kg) to IFN-$\gamma^{-/-}$ and IFNAR$^{-/-}$ C57BL/6 mice, using flow cytometry combined with confocal microscopy. Following stimulation with LPS, we didn´t find any variation of CD11b$^{+}$CD45$^{lo}$ microglial cells; however, we noticed a decrease of CD11b$^{+}$CD45$^{hi}$ (Ly6C$^{hi}$/CD11c$^{+}$) myeloid recruited leukocytes in both KO mice strains compared to their WT-treated counterparts ($p<0.05$). Unexpectedly, no significant changes were observed neither in the absolute number of MHC-II$^{+}$ cells nor in the MFI of Mi and peripheral leukocytes. Interestingly, we found an increase of CD11b$^{+}$CD45$^{hi}$Ly6C$^{+}$Ly6G$^{+}$ neutrophils from LPS primed IFNAR$^{-/-}$ mice in comparison with their IFN-$\gamma^{-/-}$ littermates ($p<0.05$). Thereby, IFNs could prove to be important players in the regulation of leukocyte recruitment to the CNS by controlling the innate immune response in neuroinflammation. Furthermore, these findings highlight the ability of a systemic TLR4-mediated challenge to signal to the CNS and alter brain´s primary immunity.

## (331) RELEVANCE OF THE BLASTOCYST CONDITIONED MEDIA ON IMMUNOTOLERANCE: FOCUS ON THE CONTROL OF THE INFLAMMATORY RESPONSE.

**ESTEBAN GRASSO, ELIZABETH SOCZEWSKI, DAIANA VOTA, LAURA FERNÁNDEZ, LUCILA GALLINO, CLAUDIA PÉREZ LEIRÓS, ROSANNA RAMHORST**

*Immunopharmacology Laboratory, IQUIBICEN, University of Buenos Aires, CONICET, Argentina.*

The decidualization of human endometrial cells involves changes in their secretome increasing the production of immunomodulatory mediators. It is associated with a sterile inflammatory response that should be later controlled to a tolerogenic microenvironment by maternal and bastocyst-derived factors. Here we focus on the production of immunomodulators during the decidualization process and we explored whether human Blastocyst Conditioned Media (BCM) could control the initial inflammatory response. As an in vitro model we used the Human endometrial stromal cell line (HESC) decidualized or not with medroxiprogesterone (10-6M)+dbcAMP (2,5 10-3M) during 8 days. We observed an increase in the production of IDO (indoloamine-2,3 di-oxigenase), CXCL8, CXCL12 and IL-1beta production ($p<0.05$ Studen T test) after the decidualization. Since IL-1beta can act as a 'double edge' mediator in early pregnancy, it is necessary for implantation but higher level display deleterious effects, we evaluated BCM effect on IL-1 production. BCM derived from human competent blastocyst reduced IL-1 intracellular production in decidualized cells, compared to BCM from blastocyst morphologically impaired. This effect was accompanied by decreased expression of ATF6 and PERK, two sensors of reticular stress, and TXNIP, a kinase/RNase associated with inflammasome activation by BCM and it was more pronounced with BCM derived from human competent blastocyst ($p<0.05$ Student t test). Finally, in an in vitro implantation model based on co-culture of blastocyst-like spheroids from trophoblast cells (BLS, from Swan-71 cell line) on decidualized-HESC cells, BLS were able to invade HESC decidualized monolayer and the BCM obtained from developmentally impaired blastocysts decreased their invasion index ($p<0.05$). In conclusion, the BCM might contribute to the cross-talk with decidualized cells controlling the inflammatory response and allowing blastocyst invasion accordingly with the blastocyst quality.

# SAI SYMPOSIUM VI

# IMMUNOTHERAPY

## ANTI-STX2 ANTIBODIES FOR THE TREATMENT OF UREMIC HEMOLYTIC SYNDROME.

**MARINA PALERMO, PHD.**

*IMEX-CONICET, Academia Nacional de Medicina, Buenos Aires, Argentina.*

## TOWARDS PERSONALIZED IMMUNOTHERAPY OF SOLID TUMORS

**STEPHEN SCHOENBERGER, PHD.**

*Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, La Jolla, California, USA*

## (533) SENESCENCE AND IMMUNOTHERAPY IN BREAST CANCER MEDIATED BY STAT3 BLOCKADE

**MARA DE MARTINO[1], MARÍA F. MERCOGLIANO[1], MERCEDES TKACH[2], LEANDRO VENTURUTTI[1], CECILIA J. PROIETTI[1], PATRICIA V. ELIZALDE[1], ROXANA SCHILLACI[1]**

*[1]Instituto de Biología y Medicina Experimental, CONICET, Buenos Ares, Argentina [2]Institut Curie, France.*

Stat3 is constitutively active in 60% of breast cancer (BC) where it promotes tumor progression and immune evasion. We described in murine BC models that Stat3 inhibition leads to a senescence program and that immunization of mice with Stat3-blocked BC cells induces an antitumoral immune response that involves $CD4^+$ Th cells and NK cells. Here, we studied the mechanism of senescence induced by Stat3 inactivation and the use of the supernatant (SN) from Stat3-blocked cells to formulate an effective immunotherapy (IT). Knockdown of Stat3 with siRNA induced senescence in triple negative (4T1, MDA-MB-231 and MDA-MB-468 cells) and ErbB2 positive (C4HD, JIMT-1 and KPL-4 cells) BC models, determined by SA-â-gal staining. Also, we observed an increase in trimethylation of histone H3 at lysine 9 and in cell cycle inhibitors expression ($p16^{INK4a}$ (p16) or $p21^{CIP1}$). Simultaneous transfection with siRNAs targeting Stat3 and p16 or $p21^{CIP1}$ reverted the senescent phenotype. Interestingly, Stat3 inhibition in vivo induced senescence and an increased p16 expression in 4T1 tumor. Then, we embedded the SN of C4HD or 4T1 cells transfected with Control siRNA (SN-Control), Stat3 siRNA (SN-Stat3) or the combination of Stat3 and p16 siRNAs (SN-Stat3+p16) in a slow delivery depot as an adjuvant of a cellular IT. Prophylactic IT before C4HD tumor challenge with SN-Stat3 and SN-Stat3+p16 decreased tumor growth (72%, $p<0.05$ and 51%, $p<0.05$ respectively vs. SN-Control). Therapeutic IT after 4T1 tumor implantation with SN-Stat3 and SN-Stat3+p16 decreased tumor growth (51%, $p<0.001$ and 41%, $p<0.01$ respectively vs. SN-Control) and pulmonary metastasis (70%, $p<0.05$ and 50%, ns. respectively vs. SN-Control). In both IT protocols the result was associated with greater cytotoxic activity of NK cells and an increase in the number of memory $CD4^+$ T cells vs. SN-Control. These results suggest that Stat3 blockade drives a senescence program in BC cells and the SN-Stat3 is an effective adjuvant for IT.

## (1066) EVALUATION OF HETEROLOGOUS PRIME-BOOST IMMUNIZATION STRATEGY AGAINST BORDETELLA PERTUSSIS USING A NOVEL OUTER MEMBRANE VESICLE BASED VACCINE AND COMMERCIAL ACELLULAR VACCINE

**GRISELDA MORENO[2], EUGENIA ZURITA[1], EMILIA GAILLARD[1], DAVID SABATER[1], MARTÍN RUMBO[2], DANIELA HOZBOR[1]**

*[1]Laboratorio VacSal -IBBM FCE UNLP CONICET y [2]IIFP-FCE UNLP CONICET, La Plata, Argentina.*

Pertussis remains an important health problem in many countries even in those with high vaccination coverage. From 1950s-1990s this disease was controlled by use of whole cell pertussis (wP) vaccines. Later in 2000s these vaccines were replaced in developed countries by acellular pertussis (aP) vaccines (purified components of *Bordetella pertussis* (Bp) absorbed to alum). The new aP vaccines, although safer, are not as effective as the wP vaccine and this has been attributed to: 1) escape form protective immunity, 2) waning immunity or 3) a failure of the aP vaccine to induced protective cellular immune responses. Under this context, we have developed a new vaccine candidate based on outer membrane vesicles (OMVs) derived from *Bp* which is capable of inducing a more robust immune response than commercial aP vaccines with a Th2/Th1/Th17 cellular profile. Here we evaluated the immunogenicity of a heterologous prime-boost regimen using OMV-base and aP vaccines. For comparison purposes homologous vaccination regimens with OMV based vaccine and aP were also performed. For all cases, the induced humoral and cell-mediated immune responses were evaluated. A robust total IgG antibodies with a high IgG2a/IgG1 ratio were detected in sera of mice primed with OMV based vaccine, suggesting that OMVs skewed the immune response to a Th1 profile. Spleen

cells from immunized mice were isolated and stimulated *in vitro* with B. pertussis antigens. Interestingly the obtained results showed that the priming with OMV vaccine induce a strong Th1/Th17 response with high values of INF-ϒ (2100 ± 350 pg/ml p<0,05), which was maintained after aP boost. In contrast, IL-5 secretion was mainly produced by spleen cells from mice primed with aP, which results in a Th2 response. The immunological characterization in the murine model of these vaccination schedules led us to propose that OMVs are highly reliable primer candidates to be considered in future as an alternative strategy against pertussis.

## (2015) EFFECTS OF DIFFERENT DRUGS IN THE RELEASE AND PROTEIC EXPRESSION OF TUMOR CELLS DERIVED EXOSOMES TO BE USED AS ACELLULAR ANTIGENS.

**FEDERICO COCOZZA[1], FLORENCIA MENAY[1], RODRIGO TSACALIAN[1], ALEJANDRINA VENDRELL[1], PURA SAMPEDRO[2], CLAUDIA WALDNER[1], CLAUDIA MONGINI[1]**

*[1]Centro de Estudios Farmacológicos y Botánicos (CEFYBO), CONICET-UBA. [2]Universidad de Morón.*

Exosomes are 40-100 nm nanovesicles released by most of cells. Tumor cells derived exosomes (Tex), used as a vaccine, elicit a specific cytotoxic response against tumor cells, with a greater immunogenicity than lysated tumoral cells. The utilization of exosomes as an easily obtainable and stable defined source of antigens is a novel technique for treating cancer. However, the amount of exosomes purified from culture cells is limited. In recent studies it was observed that cells respond to different stressors stimuli (hypoxia, acidosis, radiation, cytotoxic drugs, oxidative stress and heat shock) by releasing microvesicles. The purpose of this work was to use different stressors to enhance the exosome production, to be used as acellular immunogens for the development of an antitumor vaccine. Tex released from tumor cells in a culture of the murine T-cell lymphoma, either growing in normal or stressed conditions, were purified by differential centrifugation and ultracentrifugation. Their concentration was measured by Bradford; the purity and also the expression of exosomes protein markers, such as Hsp's, Alix and TSG-101 and tumor antigens were determined by flow cytometry and Dot Blot. It was found that cyclophosphamide cellular stress enhances the exosome production (61 ± 16) % and that these exosomes express more Hsp90 in their membrane compared with exosomes of the same cells growing without stressors. Likewise, the immune response generated by the inoculation of exosomes in normal mice was study, evaluating the humoral response by Dot Blot, as well as the survival post-challenge with tumor cells. Tex isolated from tumor cells grown with 3mM cyclophosphamide or without stress induced an immune response with a high and similar titer of antibodies in serum, previous to the inoculation with the tumor cells and this induction was reflected in the percentage of the tumor rejection in mice, without differences within the groups.

## (605) IL-2/ANTI-IL-2 COMPLEX TREATMENT INDUCES REGULATORY T CELLS AND AMELIORATES EXPERIMENTAL FOOD ALLERGY.

**PAOLA LORENA SMALDINI[1], FERNANDO TREJO[1], JOSÉ COHEN[2], ELIANE PIAGGIO[3,4], GUILLERMO DOCENA[1].**

*[1]Instituto de Estudios Inmunológicos y Fisiopatológicos IIFP, Facultad de Cs. Exactas, UNLP, La Plata, Bs. As., Argentina. [2]Laboratoire de Biologie et Thérapeutique des Pathologies Immunitaires, Centre National de la Recherche Scientifique UMR 7087, Hôpital Pitié-Salpêtrière, 756651 Paris, France. [3]Institut Curie, PSL Research University, INSERM U932, F-75005, Paris, France. [4]Centre d'Investigation Clinique Biothérapie CICBT 1428, Institut Curie, Paris, F-75005 France.*

Cow's milk allergy (CMA), mediated by an aberrant immunological reaction to cow's milk proteins (CMP), is one of the most prevalent food allergies in infants and young children worldwide. Oral and sublingual immunotherapies show promise as potential disease-modifying therapies, although no therapy has yet been approved. It has recently been demonstrated that the IL-2/ anti-IL-2 complex selectively expands regulatory T cells (Tregs). We aimed to combine sublingual immunotherapy (SLIT) using low doses of the allergen with systemic administration of IL-2/anti-IL2 to improve the safety and efficacy of the sublingual therapy Balb/c mice were sensitized with CMP and cholera toxin and then treated with PBS (Sens) or CMP (CMPdes) (sublingual) w/wo intraperitoneal injections of IL-2/anti-IL-2 (C or CMPdes/C). Mice were orally challenged with CMP and the immune response was in vitro (serum IgE, IL-5 and IFN-ã secretion by splenocytes, lamina propria Tregs, IL-10 and TGF-â) and in vivo (clinical score and cutaneous tests) evaluated. We found a lower medium clinical score in treated mice after the oral challenge with CMP, as compared with sensitized mice, with a reduction in skin mast cell reactivity in treated mice,

mainly in CMPdes/C group. Immunological changes included decreased specific IgE (2,1±0,3OD Sens vs 1,3±0.1CMPdes; 1,4±0,9C; 1,1±0,8CMPdes/C), decreased levels of Th2 cytokines and induction of IL-10 and Tregs in lamina propria of duodenum (14,6±1,2% Sens vs 28,0±0,6% CMPdes; 35,2±3,1% C; 42,5±1,8% CMPdes/C) and of sublingual mucosa of treated mice (p<0,05). The frequency of Tregs in submaxilar and sublingual mucosa was higher in CMPdes/C than CMPdes(p<0,05). However, CMPdes showed an increase of lamina propria tolerogenic dendritic cells CD11c+CD11b-CD8á+ (p<0,05). We demonstrated in a murine model of CMA that SLIT down-modulated the mucosal and systemic allergic immune response in sensitized mice and that the IL-2/anti IL-2 complex improved the sublingual immunotherapy.

## SAFE SYMPOSIUM I

## THE MEDICAL PRACTICE IN THE MULTIRESISTANT ERA

### THE LABORATORY ON THE DIAGNOSTIC AND FOLLOW UP OF RESISTANT BACTERIAL PATHOGENS WITH PUBLIC HEALTH IMPACT.

**FEDERICO NICOLA**

*Laboratorio de Bacteriología, Micología y Parasitología del Centro de Educación Médica e Investigaciones Clínicas "Dr. Norberto Quirno" (CEMIC), CABA, Argentina*

Infectious diseases are one of the most important events of morbidity and mortality in inpatients.

Microorganisms that develop antimicrobial resistance are sometimes referred to as "superbugs". New resistance mechanisms are emerging and spreading globally, (KPC, OXA carbapenemases, colistin-resistant, vancomycin-resistant, MRSA, ESBL) threatening our ability to treat common infectious diseases, resulting in prolonged illness, disability, and death. The Microbiology Laboratory has a significant role in the prevention and control of these infections and is a key element of any infection control program and is the first alert for detection of new antibiotic resistance mechanisms, outbreaks of food borne infection and intra -hospitality infections. The work of the Microbiology Laboratory covers microbial isolation and identification, determination of phenotypic and genotypic antimicrobial susceptibility patterns, epidemiological surveillance and outbreak detection, education, and quickly report of quality assured results. There are described three revolutions areas in Microbiology Laboratory in the last three decades: automatization, molecular diagnosis, and proteomic and genomics methods. These news methodologies allowed reducing the TAT, increasing the quality of the results and improve the management of infections patients.

### MULTIRRESISTANT STAPHYLOCOCCUS AUREUS INFECTIONS IN PEDIATRIC PATIENTS

**EDUARDO LÓPEZ.**

*Hospital de Niños Ricardo Gutiérrez, CABA, Argentina.*

### SEVERE INFECTIONS BY MULTIRESISTANT BACTERIA IN INMUNOCOMPROMISED PATIENTS

**NATALIA GARCIA ALLENDE**

*Servicio de Infectología, Hospital Alemán, CABA, Argentina.*

Bacteria resistant to multiple antibiotics have become a public health problem. They are associated with an increase in direct and indirect costs. Mortality rates range from 8 to 53%, due to the difficulty in the initial empirical coverage. There was a report of an increase in mortality of 7.6% per hour because of inadequate treatment. The knowledge of local epidemiology and the possibility of performing rapid tests to search multiresistant bacteria are very important to allow adjustment of antibiotic therapy in the first 24 hours.

In immunocompromised hosts, this is even more important, due to the absence of an immune system that can't cooperate with the control of infection. Different cohorts show superiority, in terms of clinical efficacy, of the combined treatment over monotherapy. Furthermore, a significant decrease in mortality is reported when meropenem, administered by prolonged infusion and at maximum dose, is included as a part of the treatment.

Acquired resistance to beta-lactams is expressed in genetic platforms associated with resistance to other families of antibiotics (such as sulfonamides, fluoroquinolones, aminoglycosides). Recently a resistance plasmid mechanism to polymyxin has been documented. This

event has direct impact on the forecast, as their presence is associated with a 4-fold increase in mortality.

It is extremely important to reduce the spread of these "superbacterias" with the establishment of surveillance institutional policies of multiresistant microorganisms, isolation contact measures and the establishment of a program of rational antibiotic use.

## SIMPOSIO I AACYTAL

## II SEMINAR ON ALTERNATIVE METHODS

## REGULATED ALTERNATIVE METHODS, A PRIORITY FOR THE REGION

### THE ROAD THAT ALLOWS BRAZIL THE CURRENT SITUATION ON ALTERNATIVE METHODS

**OCTAVIO PRESGRAVE**

*Department of Pharmacology and Toxicology, National Institute of Quality Control in Health (INCQS), Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, RJ, Brazil*

Brazil has recently approved legislation for regulating experimental animal use. On the other hand, it is a requirement that the safety of many products on the market is controlled on the basis of animal testing. Some groups at official laboratories, universities and industries are studying alternative methods, but there is no approved mechanism for funding collaborative studies, nor is there an institution responsible for coordinating these studies. These shortcomings obstruct the development of these assays in Brazil. The creation of a Brazilian Centre for the Validation of Alternative Methods (BraCVAM) would facilitate the development and validation of tests in all the institutions working on alternative methods in Brazil, and could also offer support to other Latin American countries.

### MÉTODOS ALTERNATIVOS EN ARGENTINA, ESTRATEGIAS PARA SU DESARROLLO

**MARCELO ASPREA**

*Hospital Pediátrico Juan P. Garrahan, CABA, Argentina*

Experimentar con animales de laboratorio es un tema polémico, que lleva a discusiones acaloradas y apasionadas, sus implicancias no se reducen al ámbito científico, suele extenderse a legisladores, estudiantes, industrias, opinión pública y medios de comunicación, resultando difícil permanecer indiferente ante ciertos protocolos experimentales, entonces: ¿hasta qué punto es lícito, o científica y éticamente aceptable llevar a cabo experimentos con animales? ¿En qué medida el análisis de la relación sufrimiento del animal-beneficio humano, justifica ciertos experimentos?

Con la mirada puesta en las nuevas tecnologías de Métodos Alternativos al uso del Animal de Laboratorio y con el deseo de realizar nuevos aportes, hemos lanzado una Red Regional, para integrarnos al resto de la comunidad (www.facebook.com/alternativasenred)

Desde AACyTAL (Asociación Argentina de Ciencia y Tecnología en Animales de Laboratorio), junto a AUCYTAL (Asociación Uruguaya de Ciencia y Tecnología de Animales de Laboratorio), ASOCHICAL (Asociación Chilena en Ciencias de Animales de Laboratorio) y ACCBAL (Asociación Colombiana en Ciencias y Bienestar del Animal de Laboratorio), convocamos a que se unan a ella.

La realidad nos muestra que en la región es muy poco lo realizado hasta el momento en tecnología de métodos alternativos, debido a falencias en Legislación y Regulación sobre ensayos en animales de laboratorio

La ausencia gubernamental en la promoción de esta tecnología, nos priva de una herramienta muy importante, teniendo en cuenta factores económicos que favorecerían el alcance de objetivos

El desconocimiento del usuario a estas nuevas tendencias y la dificultad de abandonar formas tradicionales en sus tareas habituales es otra de las debilidades

Consideramos contar con suficientes fortalezas para cumplir nuestra misión y una de ellas es el apoyo de las Asociaciones en Ciencias de Animales de Laboratorio de la Región, lo que facilitará la recolección de información necesaria para construir una red entre áreas de investigación biomédica, educación y desarrollo de productos biológicos, para generar una base de datos confiables

Como oportunidad para ello hemos abierto este Portal en Internet que permitirá difundir el tema y como amenaza queda la irregularidad en las Legislaciones regionales

Participación en eventos para la difusión de los métodos alternativos desarrollados en instituciones y otras estrategias que previamente se vienen desarrollando se unen a nuestro proyecto

En Mayo del 2012 convocamos desde AACyTAL a referentes, investigadores y usuarios de animales de

laboratorio a nivel nacional a realizar 3 Workshops para la elaboración de un Proyecto de Ley, el cual recientemente ha sido presentado a la Legislación y que en su capítulo 16 contempla el uso de MA

Sumando todas las herramientas anteriores se presentó en Abril 2015 al SNB / MinCyT un proyecto nacional para organizar la Red Nacional de Métodos Alternativos (RAMA), dicha solicitud derivó a que en Junio del 2016 hayamos sido indicados como referentes del tema para trabajar junto al RECyT (Reunión de especialistas en Ciencia y Tecnología Plataforma Mercosur) para fomentar el desarrollo de los Métodos Alternativos en Argentina

Dentro de los desarrollos de MA en Argentina, podemos mencionar:

Tecnología IgY: producción y uso de anticuerpos de yema de huevo, donde el uso de anticuerpos de aves presenta varias ventajas: el costo de producción es relativamente más bajo que en mamíferos y se pueden producir en grandes cantidades, permitiendo su aplicación en el desarrollo de estrategias de inmunoprofilaxis e inmunoterapia. Algunas de estas aplicaciones son la prevención y tratamiento de diarreas humanas y animales, caries, xenotransplantes, síndrome urémico hemolítico, fibrosis cística, elaboración de antivenenos, etc. no presentando reacciones cruzadas con los factores reumatoideos o los anticuerpos humanos anti-ratón, ni activan el sistema de complemento mamífero

Monitoreo Endócrino No-Invasivo en Orina y Heces de Mamíferos, la extracción de muestras de sangre constituye, en sí mismo, un procedimiento que puede modificar los niveles hormonales. Una alternativa eficiente es el monitoreo no-invasivo de metabolitos de hormonas esteroideas excretadas en diversas matrices como materia fecal, orina, saliva, pelos , donde se pueden estudiar aspectos tan diversos como los ciclos reproductivos, variaciones estacionales en las concentraciones hormonales, diferencias sexuales y de comportamiento asociadas a hormonas, asociación entre posiciones jerárquicas, función tiroidea, efectos de tóxicos ambientales sobre la función endocrina, estrés y concentraciones hormonales con efectos sobre la reproducción, así como efectos de las actividades humanas sobre el bienestar animal

*Galleria mellonella* para sustituir a los modelos vertebrados en investigaciones científicas, en los últimos años, ha aumentado el uso de larvas de insectos en experimentos científicos para sustituir la demanda de pequeños mamíferos. En particular, *Galleria mellonella*, la “polilla grande de la cera” ha mostrado ser un modelo animal apto para reproducir algunas infecciones con un comportamiento patológico e inmunológico muy parecido al que se puede observar en los mamíferos. Este modelo reduce significativamente todo tipo de costos, incluyendo: instalaciones edilicias, alimentación, espacio para manipulación, controles de higiene y de homogeneidad genética, entre otros

Receptores Cys-loop en el organismo, modelo *Caenorhabditis elegans*, los receptores pentaméricos de la familia Cys-loop intervienen en procesos tales como transmisión neuromuscular, aprendizaje, cognición e incluyen al receptor nicotínico (AChR), de serotonina, de GABA y de glicina. El nematodo de vida libre *Caenorhabditis elegans* es un buen modelo para el estudio de comunicación neuronal y patologías humanas asociadas porque la transmisión sináptica y en particular los receptores Cys-loop se conservan con los de vertebrados. Mediante el análisis de las propiedades electrofisiológicas en cepas mutantes nulas para diferentes subunidades nicotínicas se descifra la composición de este receptor y se determina el rol funcional de cada una de sus subunidades. Se han generado y caracterizado cepas transgénicas conteniendo mutaciones que imitan las encontradas en síndromes miasténicos congénitos humanos. Se ha demostrado que es posible reproducir los cambios funcionales observados en los pacientes, por lo que *C. elegans* es un modelo válido para estos desórdenes musculares

Drosophila como modelo para identificar genes involucrados en neurodegeneración, en la última década Drosophila ha cobrado impulso como sistema modelo para desentrañar las bases moleculares de la neurodegeneración, el cáncer, la inmunidad innata y el envejecimiento. En ese contexto se han desarrollado distintos modelos que recrean diversos aspectos de la enfermedad de Alzheimer, Parkinson y Hungtinton, entre los cuales se basan en la sobre-expresión de variantes silvestres o mutadas de genes causales de enfermedad en el hombre. Se llevó a cabo un rastrillaje genético por desregulación de la expresión de genes al azar basado en un ensayo automatizado de actividad locomotora en dos estadios de la vida de la mosca *Drosophila melanogaster*. Como resultado de este esfuerzo se ha identificado varios loci que afectan específicamente este comportamiento en moscas de mediana edad, y uno de ellos, enabled, recrea tres de las características propias de enfermedades neurodegenerativas: la aparición tardía de síntomas, la progresión y la vulnerabilidad específica de ciertas poblaciones

Preservación de tejidos y simulación, el entrenamiento de personal médico quirúrgico tradicionalmente implicó la utilización de un número considerable de animales, a través de nuevos métodos alternativos se ha obtenido inmejorables beneficios en la adquisición de capacidades en la curva de aprendizaje y consecuente reducción del uso de animales para tales prácticas, mereciendo un capítulo aparte la Simulación, que consiste en un proceso de diseñar un modelo de un sistema real y llevar a término experiencias con él,

Nos encontramos, por tanto, en un punto de inflexión. La Ciencia y la Sociedad está avanzando respecto a hace unos años, pero aún quedan muchos puntos por cubrir y muchos obstáculos por superar. Por ello, es importante que no cesen los esfuerzos por parte de la comunidad

científica para que apoyen los métodos alternativos, para que se siga invirtiendo en su desarrollo e implementación en todos los ámbitos, ya que de este modo todos nos beneficiaremos: los humanos porque desarrollaremos modelos más fiables (Por ej: se está investigando sobre la metabolización de fármacos en cultivos de hepatocitos humanos y se están obteniendo resultados más fiables que en otras especies, cosa, por otra parte, lógica y esperable) y los animales no humanos, porque se evitaran sufrimientos innecesarios.

Pero hasta que la sociedad logre este noble fin... ¿Cuántos errores vamos a cometer por la prescripción de fármacos a humanos? ¿Cuántos animales deberán sufrir en silencio en los laboratorios? De nosotros depende

## STRATEGIES TO REDUCE AND REPLACE IN VIVO EVALUATION OF OCULAR IRRITATION

**SUSANA GORZALCZANY**

*Pharmacology Chair, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires*
*Junín 956 Piso 5, Ciudad Autónoma de Buenos Aires*
*sgorza@ffyb.uba.ar*

The eyes are constantly being exposed to different substances. The exposure can be incidental, accidental, or intentional, with cosmetics, household products or drugs. For that reason, the evaluation of the potential irritation that, those products could be induced in eyes is essential to give evidence that their use is safe for human health. In this sense, in forties, Draize test sprang as the only assay to analyze the effect of different substances on or around the eyes. Rabbits are often preferred for their large eyes with well-described anatomy and physiology, ease of handling and availability. The eyes of rabbits are generally more susceptible to irritating substances than the eyes of humans but many exceptions do exist. However this procedure has been criticized because of the potential injuries on the eyes of the rabbits and for the subjectivity in the scoring system to measure the magnitude of the lesion produced by the consumer products.

The 3 Rs (replacement, refinement and reduction of the use of animals) derive from the humanitarian intent of minimizing unneccessary discomfort of the laboratory animals. Alternatives to the Draize eye test have been proposed. A refinement alternative aims to lessen animal distress, a reduction alternative decreases the number of animals used in testing and the replacement alternatives intend to eventually do away with whole-animal testing.

Different systems were used to evaluate the effect of substances in isolated environments devoid of hormonal, immune or neural influence, although the elimination of other biological factors does not allow the method to mimic interactions occurring in the whole organism, particularly with a specialized organ such as eye. Since the early 1980s, many *in vitro* methods have been developed, including isolated organ methods, organotypic models, reconstituted human tissue models, cell-based cytotoxicity methods, and cell function-based assays.

Although no single in vitro test has emerged as being completely acceptable for full replacement, various strategies are being used in order to contribute toward refining and reducing animal experiments.

# NANOMED SYMPOSIUM
# A GLIMPSE INTO THE LANDSCAPE OF NANOMEDICINES IN LATINO AMERICA

## CHRONIC DISEASES, THERAPY, DIAGNOSTIC AND THERANOSTICS BY USING NANOPLATFORMS

**MARCELO KOGAN**

*Department of Pharmacology and Toxicology. Faculty of Pharmacy. Universidad de Chile. Center for Advanced Study*

The advent of nanotechnology has radically changed the way we diagnose, image and treat diseases, with novel nanoplatforms capable clinically important functions, including detecting cancer at its earliest stages and location, as well as delivering anticancer therapeutics specifically to tumor cells. The nanotechnology approach to cancer has focused on three main avenues: early detection; imaging for diagnostics or assessment of targeted delivery. Also multifunctional therapeutics are of interest, whereby nanoplattforms are loaded with multiple functional moieties capable of selective targeting, imaging and delivery of specific drugs to malignant cells (1,2,3). In relation with this is possible to mention the so called

theranostics which consist in the diagnostic and treatment of pathologies in a unique procedure (4) .

In the talk will be discussed the potential use of different nanomaterials multifunctionalized with different biomolecules for cancer theranostics, diagnostic *in vitro* for the ultrasensible detection of biomarkers and nanoplattorms for drug delivery. The state of the art of clinical applications of nanomaterials in diagnostic and treatment will be commented.

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***Acknowledgements***

Projects Fondecyt 1130250 and Fondecyt-FONDAP 15130011

## WITH THE FOCUS IN MACROPHAGES: INHALABLE AND ANTI-ATHEROGENIC NANOMEDICINES.

**EDER ROMERO**

*Nanomedicine ResearchProgram, Universidad Nacional de Quilmes, Buenos Aires, Argentina*

The targeted delivery is one of the most popular promises of the nanomedicine. A promise however, that suffers from a number of limitations: inpharmacodynamics, those imposed by the restricted accessibility to the target site and the dependence of the convective extravasation; in security, because of the addition of exogenous ligands is frequently reactogenic-; in stability, because the higher the construct complexity, the more labile to physical chemical, enzymatic damagesand shear stress during storage or administration. Targeted nanoparticles are difficult to be scaled up since the addition of chemical bonds results in lengthy and expensive procedures. In a technical field where the product is the process, the artificial decoration of nanoparticles results in products difficult to be reproduced at high scale and also of challenging full structural characterization.

In this scenario, the development of new colloidal nanoparticles offering new functionalities such as a high endurance to casual manipulation and storage conditions, plus targeted delivery, with no aid of expensive additives or chemical derivatizations, is of utmost industrial relevance. To get these achievements however, the classical phospholipids employed to prepare nanoliposomes, (the massively accepted by the pharmaceutical industry nanoparticles), has to be replaced by a new type of building block, quite phylogenetically distant from phospholipids extracted from animals, plants, fungi or bacteria: the archaeolipids.

In this presentation I will address the potentialities of two of our most recent engineered nanoparticles prepared on the bases of archaeolipids (archaeosomes) at our Nanomedicine Research Program-2: inhalable pH sensitive archaeosomes for targeted delivery of anti-inflammatories to alveolar macrophages and long circulating savage archaeosomes for targeted delivery of bisphosphonates to macrophages of atheromatous plaque.

## INNOVATIVE APPROACHES TO DECORATE THE SURFACE OF POLYMERIC NANOCAPSULES.

**ADRIANA POHLMANN**

*Department of Organic Chemistry, Institute of Chemistry, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil*

Biodegradable nanocarriers have been studied as a promising alternative to therapeutics contributing to expand the applications of nanotechnology. The control of size distribution, by using self-assembly methods of preparation, affects the drug biodistribution and release. Some advantages of the nanoparticulate systems are related to the drug targeting reducing side effects and increasing therapeutic index. The presentation addresses the aspects of the synthesis of lipid-core nanocapsules, an original type of carrier useful to encapsulate poorly water-soluble drugs, as well as their surface functionalization using an innovative approach based on an organometallic complex. Examples of physico-chemical characterization and biological applications of surface-functionalized lipid-core nanocapsules are discussed: i) LDL(-) recognition and ii) Mucopolysaccharidosis type I. In summary, this presentation shows that self-assembled nanoparticles are promising devices for drug delivery and targeting. (CNPq, CAPES, FAPERGS).

## NANOMEDICINES FOR THE TREATMENT OF INFLAMMATORY BOWEL DISEASES.

**HIGA L, JEREZ H, ROMERO E, MORILLA MJ.**

*Nanomedicine ResearchProgram, Universidad Nacional de Quilmes, Buenos Aires, Argentina*

Inflammatory bowel diseases such as Crohn's disease and ulcerative colitis, are chronic relapsing disorders of the gastrointestinal tract, characterized bychronic inflammation and epithelial injuryinduced by the uncontrolled activation of the mucosal immune system. Dendritic cells and macrophages are key cells in the inflamed mucosa, which produce large amounts of pro-inflammatory cytokines. The current treatment is symptomatic, but the frequent oral intake of anti-inflammatory and immunosuppressant drugs or the systemic administration of biological agents such as the anti-TNF antibody infliximabis poorly effective and cause serious adverse effects.

More efficacious and safer therapies could rely on developing macrophages-targeted nanoparticles capable of specifically delivering high doses of immunosuppressantor anti-inflammatory drugs with minimal exposure of healthy. To that aim, we developedarchaeolipid nanoparticles made of a core of solid lipid and a shell of total polar archaeolipids (TPA) extracted from the halophilic archaebacterial *Halorubrumtebenquichense.* TPA are a mixture of saturated isoprenoid chains linked via ether bonds to the glycerol carbons at the *sn*2,3 position. In contrast to conventional phospholipids, TPA are hydrolytic, oxidative and enzymatic attack resistant. Besides, TPA are ligands for the macrophages scavenger receptors class A. Archaeolipid nanoparticles would combine high resistance under gastric tract with extensive uptake by macrophages.

Overall, our studies showed that ultra-small, highly negatively charge mucopenetratingarchaeolipid nanoparticles loaded with dexamethasone, but no nanoparticles lacking TPA, resulted highly stable under gastrointestinal conditions, were highly up taken by macrophages and reduced the secretion of pro-inflammatory cytokines from macrophages stimulated with lipopolysaccharide. We consider that archaeolipid nanoparticlescould improve the current therapies of inflammatory bowel diseases.

## DESIGN AND DEVELOPMENT OF NEW NANOTECHNOLOGICAL PLATFORMS FOR PHARMACOTHERAPY

**SANTIAGO PALMA**

*Pharmaceutical Technology Research and Development Unit (UNITEFA) (CONICET); Pharmacy Department, Faculty of Chemical Sciences, NationalUniversity of Cordoba (UNC), Córdoba, Argentina.*

The use of active surface compounds (surfactants) in pharmaceutical technology has been widely explored. It is well known that surfactants at determined concentrations (minimal aggregation concentration) begin to form aggregates as consequence of the increment of interactions between molecules. As concentration is raised, the interactions between adjacent structures are increased leading to the coalescence of the system in larger structures usually denominated liquid crystals. The formation of such structures can be evidenced through noticeable changes in viscosity, conductivity, birefringence and X-ray diffraction patterns. The different liquid crystal systems formed from surfactant-solvent interactions are defined as lyotropic liquid crystals (LLC).

From several years ago we have been studying a group of polar lipids consisting of alkyl vitamin C derivatives (ASCn). Their amphiphilic nature allows these compounds to form aggregates, mainly lamellar mesophases. The performed studies allowed us to evaluate the potential utility of this new liquid crystal system, which have evidenced very interesting properties as pharmaceutical carrier. In this lecture, we described the general properties of these systems and the results concerning to their potential useful for different applications.

The nanostructured systems derived from the self-assembly properties of ASCn showed appropriated characteristics as pharmaceutical platform for drug delivery, especially through administration routes where permeation enhancement is necessary.

## DESIGN OF SILICON OXIDE NANOPARTICLES. STUDIES OF THE INTERACTION WITH CELL SYSTEMS AND DRUG TRANSPORT.

**MARTIN DESIMONE**

*IQUIMEFA-CONICET, University of Buenos Aires, CABA, Argentina.*

The application of silica nanoparticles in the biomedical field experienced a great development in recent years. The driving forces for these and future developments are the possibility to design nanoparticles with homogeneous size and structure amenable to specific grafting. Indeed, it is possible to tune the characteristics of the silica nanoparticles to meet the requirements of each specific cell and desired application. Moreover, the effect of silica particle surface functionalization on antibiotic sorption was first studied, enlightening the role of electrostatic and hydrophobic interactions. Finally, core–shell silica particles were prepared allowing for the dual delivery of gentamicin and rifamycin.

## TWO IN ONE: MULTIFUNCTIONAL NANOPARTICLES FOR THE TREATMENT OF BREAST CANCER.

**MARÍA INÉS DIAZ BESSONE[1], LORENA SIMÓN GRACIA[2], PABLO SCODELLER[2], GALO SOLER ILLIA[1], TAMBET TEESALU[2], MARINA SIMIAN[1]**

*[1]Nanosystems Institute -University of San Martin, Argentina, [2]Laboratory of Cancer Biology, University of Tartu, Estonia*

Seventy five percent of breast tumors express estrogen receptors. Tamoxifen, a selective estrogen receptor modulator, is the most widely used therapy for these patients. However, about one third of treated patients eventually developstamoxifen resistance and cancer reappears. We previously showed that fibronectin, when bound to cell surface β1-integrins,induces tamoxifen resistance in breast cancer cells. Moreover, we found that tamoxifen leads to an increase in breast cancer stem cells that are positive for β1-integrin. Nanoparticles (NPs) provide new features and functions that aredifferent from those present in the individual components. In particular, small size, high surface area/volume ratio and multifunctionality, make NPs attractive as carriers for drugs and diagnostics. The aim of this project was to investigate the effectiveness of multifunctional tamoxifen –loaded NPs,compared to free tamoxifen, for the treatment of breast cancer. To do so we to designed iRGD-coated and tamoxifen –loaded polimersomes and tested their efficacy on MCF-7 breast cancer cells in vitro and in vivo. Our results show that at equivalent concentrations, tamoxifen has a greater effect on cell viability when carried in NPs ($p<.001$). iRGD-coated tamoxifen NPs reduce stem cell growth ($p<.01$), contrary to what is observed with free tamoxifen. Cell uptake of NPs is increased when they are coated with iRGD, as well as in vivo tumor homing. Preliminary results show increased survival of mice carrying MCF-7 tumors when treated with iRGD-coated tamoxifen NPs, compared to free tamoxifen controls. Our results suggest that iRGD-coated tamoxifen NPs could be a rationale alterative for the treatment of estrogen receptor positive breast cancer, leading to increased disease free survival.

## LIPOSOMES FOR TOPICAL TREATMENT OF CUTANEOUS LEISHMANIASIS.

**DOLORES C. CARRER, MA. FLORENCIA PERALTA**

*Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC-CONICET-UNC, Córdoba, Argentina*

Cutaneous Leishmaniasis is an endemic disease that affects millions of people worldwide. It is one of the five most widespread orphan diseases in Latin America in particular. In Argentina, it has traditionally been found mainly in the tropical/subtropical regions in the North-East of the country. This is however changing, with the vector and the disease being found, in the last few years, also in regions further South in the country and reaching the province of Córdoba. The reasons for the spreading of the disease are multiple and not straightforward to control. I will discuss the available methods for treatment and the new treatments being developed, in particular topical treatments. I will briefly present our own efforts in the field.

## NANOTECHNOLOGYCAL TOOLS EMPLOYED TO IMPROVE THE OPHTHALMOLOGIC THERAPY.

**DANIELA QUINTEROS**

*Pharmacy Department, Faculty of Chemical Sciences, National University of Cordoba (UNC), Córdoba, Argentina*

Acetazolamide (AZM) is a carbonic anhydrase inhibitor, mainly used to reduce intraocular pressure in the treatment or long-term management of glaucoma. However, the potential of topical treatment is limited, due to its low permeability in ocular epithelium. An alternative to overcome this limitation is the incorporation of AZM in nanopar-

ticulate systems, such as polymeric nanocapsules (NC). In this way, this work aimed to prepare, characterize in vitro and in vivo AZM-loaded NC using ethylcellulose (EC) and Eudragit® RS100 (EUD) as encapsulating polymers. These NCs showed very high encapsulation efficiency, they were physically stable and their size was around 220-110 nm for NCEUD and NCEC, respectively. Due to the chemical characteristics of the polymers, NCEUD possesses positive surface charges, whereas for NCEC, the zeta potential was negative. In vitro release studies showed a characteristic release pattern corresponding to a classical behavior observed for devices based on a reservoir, where the properties of the polymer membrane modulate drug release. In both cases, the AZM release was practically independent regarding its concentration. Ex vivo assays, where the permeation through isolated cornea was studied, evidenced that the amount of AZM permeated from EC and EUD nanoparticles was quite higher than in the case of AZM solution. We hypothesized that nanoparticles may work as a carrier facilitating drug penetration across the cornea. Besides, the very small size and the mucoadhesive properties of NCs, particularly NCEC, could favor a close contact with the cornea surface facilitating its penetration. In vivo studies, related to the hypotensive effect of NCs in normotensive rabbits, showed that NCEC formulation was the most efficient, since an increased amount of permeated drug was observed, along with a greater IOP decrease and longer duration of the effect. This novel formulation could be a promising alternative for a more efficient treatment of glaucoma.

## INDOCYANINE GREEN WITHIN POLYMERIC MICELLES AS POTENTIAL IMAGE AGENTS TO MAP SENTINEL LYMPH NODES

**NICOLE LECOT[A], MARCELO FERNÁNDEZ LOMONACO[A], PABLO CABRAL[A] AND ROMINA GLISONI[B]**

*[a]Laboratorio Radiofarmacia, Centro de Investigaciones Nucleares, Facultad de Ciencias, UdelaR, Montevideo, Uruguay.*
*[b]NANOBIOTEC UBA-CONICET, Department of Pharmaceutical Technology, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Buenos Aires, Argentina.*

The image-guided surgery (IGS) has been widely used in clinic to map sentinel lymph nodes (SLN) of primary tumors from breast, skin, colorectal, lung and other sites. IGS require a near infrared (NIR) fluorescent contrast agent to locate the position of SLN. A sentinel lymph node biopsy (SLNB), is a procedure in which SLN is identified, removed, and examined to determine whether cancer cells are presented. A positive SLNB result indicates that cancer is present in SLN and possibly may be present in other regional lymph nodes and organs. NIR indocyanine green (ICG) is a FDA-approved water soluble fluorophore, used for various diagnostic purposes for example IGS/SLNB. ICG tends to the self-aggregation in aqueous solution to form dimers, oligomers and polymers, depending the concentration used. As a result of this, the fluorescence quenching of ICG often occurs, reducing the fluorescence efficacy. On the other hand, due to the strong protein-binding and the fast uptake to the liver, ICG displayed only a half-life of 3-4 minutes when injected intravenously. The aim of this work was to develop a novel micellar formulation containing ICG with improved physicochemical properties such as: *(i)* an optimal fluorescence, *(ii)* a better stability in aqueous medium and *(iii)* a longer half-life in circulation, using polymeric micelles (PMs), made of pristine poly(ethylene oxide)-*b*-poly(propylene oxide) block copolymers (PEO-PPO) and their glucosylated derivatives, and finally to evaluate these nanocarriers loaded with ICG intradermally, in an *in vivo* assay against the commercially available free ICG in solution.

## 3D IN VITRO TISSUE MODELS IN NANOMEDICINE RESEARCH

**PRISCILA SCHILRREFF, MORILLA MARIA JOSE, EDER ROMERO**

*Nanomedicine Research Program, C3R, Universidad Nacional de Quilmes, Buenos Aires, Argentina*

With the increasing numbers of new nanomedicines there is a need to investigate their possible health adverse effects in humans.Nanomedicines can potentially be administered through a number of routes, including oral, dermal, pulmonar and ocular. Extensive*in vitro* and *in vivo*research is needed before nanomedicines will be suitable for medicinal use. For that purpose, millions of animals are used. However, 95 percent of new drugs fail because they do not work in humans or are unsafe, despite previously appearing safe in preclinical animal tests. According to US FDA, adverse drug reactions cause over 100.000 deaths annually. Besides, *being*costly and poor predictive of human toxicity due to inter-species differences, animal tests can *be cruel* and painful. Therefore,

the replacement of animals with *in vitro* human cell-based models is required. In this context, 3D *in vitro*models provides a cellular microenvironment that preserves cell-cell interactions, function and tissue architectureand could be a useful tool for *predicting*the effects of *nanomedicines* in humans.

A number of celular 3D models have been developed including skin, liver, cardiac, pulmonar, corneal and ephithelial intestinal tissues. This presentation will focus on technicalspecifications for the development of intestinal, normal and disease skin models usedfor the evaluation of toxicity, penetration and efficiency of new nanomedicines.

## DENDRITIC THERMORESPONSIVE NANOGELS AS VERSATILE PLATFORMS FOR BIOMEDICAL APPLICATIONS

**MARIA MOLINA[1,2], MARCELO CALDERÓN[2]**

*1:Universidad Nacional de Rio Cuarto. Rio cuarto, Argentina, 2:Institut für Chemie und Biochemie, Freie Universität Berlin. Berlin, Germany*

Nanogels are nanosizedcrosslinked networks composed of hydrophilic or amphiphilic polymer chains. They are developed as carriers to transport small molecules such as drugs, dyes, or biomacromolecules. Thermoresponsive polymers undergo a phase transition at a certain temperature in aqueous media. As a consequence,they can change their aggregation state, exhibit conformational change and undergo shrinking, swelling, or micellization upon a thermal trigger. The combination of nanogel properties and thermoresponsiveness represents a promising approach for the development of smart nanocarrier systems, which reveals high loading capacity, improves drug stability, and thus can be used for stimuli-controlled release in drug delivery. Herein, the engineering of thermoresponsive dendritic nanogels using different thermoresponsive polymers and synthetic methodologies for biomedical applications is presented.

## NANOMATERIALS AND CELLS: INTERACTIONS AND EFFECTS.

**ALICIA LORENTI**

*Instituto de Ciencia y Tecnología Dr. Cesar Milstein, Fundación Pablo Cassara, CABA, Argentina*

Tissue engineering is a field of Regenerative Medicine that combines cells, biomaterials, and suitable biochemical and physicochemical factors in order to obtain substitutes to improve or replace biological damaged tissues. The interactions between cells, extracelular matrix, and their microenvironment play key roles in controlling the cell fate (differentiation/dedifferentiation, proliferation, adhesión, spreading, migration, apoptosis).

Progress in the development of nanotechnology has stimulated the applications of nanomaterials for regenerative medicine/tissue engineering. However, thinking that a cell is a particle composed by nano compartments (cell membranes and nuclear, surface proteins, genetic material, cytoskeleton), the interactions between nanomaterials and cells should be taken in consideration when considered any tissue engineering development, even when these interactions are not well understood yet. Nanomaterials are not a simple miniaturization of macroscopic counterparts; they exhibit distinctive physical, chemical, optical, and mechanical properties.

The understanding of nanomaterial-cell interactions will facilitate improved biomaterial design for a range of biomedical and biotechnological applications. In this sense, the effects of the shapes, kind of surfaces, and chemical functionality of nanomaterials on cellular processes need critical evaluation, in order to understand the true nature of biological effects. Moreover, the intracellular safety concern of the nanomaterials as a result of its cellular uptake, its intracellular fate and degradation, and its influence on toxicity remains to be clarified in detail.

## NANOTECHNOLOGICAL COLLAGEN SCAFFOLDS FOR DERMAL REGENERATION

**HELENA PARDO[1], LUCIANA PEREIRA[1], ANALÍA CASTRO[1], ÁLVARO W. MOMBRÚ[1], RICARDO FACCIO[1], NATALIA ODDONE[2], JUAN C. BENECH[2], CRISTINA TOURIÑO[3], JUAN PABLO VILLANUEVA[1], PATRICIA ZIMET [1], PABLO MIRANDA[1], MARIANO ROMERO[1], INÉS ALVAREZ[4], HÉCTOR PÉREZ CAMPO[4]**

*[1]- Centro NanoMat, DETEMA, Facultad de Química, Universidad de la República, Montevideo, Uruguay; 2- Laboratorio de Nanobiología y Señalización Celular, Instituto de Investigaciones Biológicas Clemente Estable, Ministerio de Educación y Cultura, Montevideo, Uruguay; 3-Departamento Básico de Medicina, Hospital de Clínicas, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay; 4- Instituto Nacional de Donación y Trasplante, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay.*

The treatment of hard-to-heal acute and chronic wounds represents a critical and expensive issue for medicine. In this regard, engineering skin substitutes arise as an alternative treatment suitable for dermal

regeneration, as they resemble structural and functional characteristics of the native extracellular matrix. Therefore, the main aim of this work is to develop a type I collagen scaffold which incorporates L-ascorbic acid (AA) loaded chitosan nanoparticles (CSNp) in order to improve the wound healing of the skin. The AA promotes the natural tissue collagen synthesis.

In order to develop a porous scaffold, type I collagen was obtained by chemical degradation of bovine tendon. The primary crosslinking was performed by the addition of native bovine chondroitin sulphate, afterwards the scaffolds were frozen and irradiated with gamma rays. Then, a medical grade silicone layer was adhered to the scaffolds, resulting in a bilayer material. Finally, the AA-CSNp were incorporated by inmmersion. The final product was characterized physically and chemically through: Scanning electron microscopy, atomic force microscopy and mechanical properties test by texturometry. The biologically cytotoxicity was assessed in vitro and in vivo.

The AA-CSNp were prepared by the ionic gelation technique and characterized using transmission electron microscopy and dynamic light scattering. Cell viability studies have been carried out in order to study de citotoxicity of the CSNp. Through this technique, were obtained monodisperse and spheric AA-CSNp with an average size of 109 nm, a 20 mV Z potential and 21 % of encapsulation efficiency.

15kGy gamma irradiated before lyophilisation collagen scaffolds has shown the best mechanical properties compared with other doses of gamma rays. This radiation also renders them sterile. The collagen scaffolds obtained had not shown cytotoxicity during in vitro studies. From in vivo results it has been observed an improvement in the skin healing.