ICGEB International Centre for Genetic Engineering and Biotechnology | Developing Knowledge

ICGEB Research Grants Programme

RESEARCH GRANTS COMPLETED in 2022

CRP - ICGEB RESEARCH GRANTS COMPLETED IN 2022

COUNTRY	PRINCIPAL INVESTIGATOR	PROJECT TITLE
ARGENTINA	Javier PALATNIK	Control of cellular programs by microRNAs in plants
BOSNIA AND HERZEGOVINA	Violeta SOLJIC	Understanding the incomplete extravillous trophoblast invasion in pregnancies complicated with severe and non-severe preeclampsia
BRAZIL	Marta GIOVANETTI	One Health International Network for SARS-CoV-2 real time genomic monitoring (*funded by IILA*)
CHILE	Leandro J. CARRENO	Targeting type-I and type-II natural killer T cells to enhance effector and memory B cell responses *(Early Career Return Grant*)
COSTA RICA	Rodrigo MORA-RODRÍGUEZ	Development and validation of a genetically encoded fluorescent reporter for Dengue and Zika virus NS2B-NS3 protease activity and its application to explore the phenotypic diversity of viral infection in terms of replication,
HUNGARY	Daniel HILLIER	Broad and efficient gene expression via systemic gene delivery of engineered AAVs in a preclinical species
KENYA	Wilton Mwema MBINDA	Generating blast resistant Finger Millet plants through gene-specific genome editing
MALAYSIA	Mohd Kaisan Bin MAHADI	Vagus nerve stimulation: Investigation on cholinergic anti-inflammatory activity and macrophage polarization in rats with induced myocardial infarction *(Early Career Return Grant*)
MEXICO	Hilda Minerva GONZÁLEZ SÁNCHEZ	Human germinal center organoids as a tool to understand B cell biology and for the identification of monoclonal antibodies with therapeutic potential against SARS-CoV2 (*Early Career Return Grant*)
RUSSIA	Alexander GABIBOV	QM/MM Approach for remodeling of biocatalytic specifity
SLOVAKIA	Andrej KOVAC	The tryptophan kynurenine pathway-theraputic strategy for neuroprotection in tauopathies
SLOVENIA	Iva HAFNER BRATKOVIČ	Sonogenetics: ultrasound-inducible transcriptional regulation
SRI LANKA	K.P. Renuka ATTANAYAKE Nilmini	Genetic dissection of polyethylene degradation ability of *Perenniporia* sp. isolated from decaying hard woods in Sri Lanka
TANZANIA	Jaffu Othniel CHILONGOLA	Inter-epidemic Transmission Dynamics of Rift Valley, Dengue and Chikungunya viruses in Tanzania
TRINIDAD AND TOBAGO	Rajini HARAKSINGH	Genetic contributors to Diabetes Mellitus in Indo-Trinidadians (*Early Career Return Grant*)
URUGUAY	Federico BATTISTONI	Decoding the molecular interaction between the plant growth promoting bacteria *Streptomyces* sp. UYFA156 and it host *Fescue arundinacea*

URUGUAY	Álvaro DÍAZ	Novel mechanisms of inflammatory macrophage proliferation
VIET NAM	Xuan-Hung NGUYEN	Identification of target autoantigens for narcolepsy type I *(Early Career Return Grant*)

ARGENTINA

Title: Control of cellular programs by microRNAs in plants
Principal Investigator: Javier Palatnik, RNA Biology Lab, Instituto de Biología Molecular y Celular de Rosario (IBR), 2000 Rosario, Argentina. Tel: +54-341-4237070
ICGEB Contract No.: CRP/18/004
ICGEB Reference No.: CRP/ARG17-01
Abstract: microRNAs (miRNAs) are essential endogenous regulators of gene expression. They are generated from foldback precursors by the microprocessor, whose main component is DICER-LIKE1 (DCL1). The mature miRNAs of ~21 nt length are incorporated into ARGONAUTE complexes, which they guide to target mRNAs, which are subsequently cleaved or translationally arrested. While the current scenario involves the recognition of the precursor structure by DCL1 during miRNA biogenesis, we found that in addition there are key sequence features essential for their processing. miR396 is an evolutionarily ancient miRNA, which in Arabidopsis and other angiosperms, regulates GROWTH-REGUALTING FACTORS (GRFs), which are plant-specific transcription factors known to promote plant growth and organ size, including grain size in crops.
Most of the GRFs in angiosperms have a perfectly conserved sequence that serves as a miR396-binding site, albeit, in some cases this regulation could have been lost. One of these cases is the Arabidopsis GRF5, which we found to be transcriptionally repressed by ARF2 in a complementary mechanism to miRNA miR396.
The miR396-GRF module has been implicated in the control of cell proliferation, cell size and stem cell maintenance. In addition, the miR396-GRF system can be a valuable tool in plant regeneration, which can be useful for crop improvement.
Objectives:
(i) To understand the molecular mechanisms underlying the control of cellular programs by miR396;
(ii) To determine the role of microRNAs and other small RNAs during cellular reprogramming;
(iii) To evaluate the improvement of plant yield by modulation of the miR396-GRF network.
Results Obtained:
We studied the role of the primary sequence in the recognition of the miRNA precursors. These results are important to understand the regulation of miRNAs in general, including miR396. We found that, at least during plant miRNA biogenesis, a C-C mismatch pair along a double stranded region of miRNA precursor is detrimental for its processing. A molecular dynamics analysis showed that C-C bases are in continuous movement (shear), while Terbium (III) probing, which generates cuts at flexible RNA segments, revealed that a dsRNA containing a C-C mismatch is more flexible than one harbouring a C-U mismatch. Taken together, these results show that mismatches formed by different nucleotides can affect the structural properties of the foldbacks, which in turn is reflected in a differential miRNA processing. Other mismatches such as G-G, A-G/G-A are also found at low frequency in plant miRNA precursors and, at least in certain contexts, also impaired miRNA biogenesis.
Furthermore, our results indicate that post-transcriptional control of the GRFs through an evolutionarily conserved miR396-binding site is a highly common scenario in plants; however, some GRFs display changes in the miR396-binding site that might modulate the miRNA-dependent control or abolish this regulation entirely. Brassicaceae species share a group of GRFs that lack miR396 regulation, including Arabidopsis GRF5 and GRF6. We found that the transcription factor ARF2 binds directly to the GRF5 promoter and repress its expression. Most interestingly, the mutation of this evolutionarily conserved region in the GRF5 promoter mimics mutations in miR396-binding site of the miRNA-regulated GRFs, as they both cause the ectopic expression of the transcription factors in a broad range of tissues.
We identified and characterised wheat plants with changes in the miR396-binding site of eleven GRFs. These are tilling mutants, and therefore, they are not considered transgenic or genetically modified plants, and can be used directly in breeding programs. We are further exploring two promising lines to assess their biomass and grain size.
Most interestingly, in collaboration with Jorge Dubcovsky (UC Davis, USA), we found that a fusion protein combining wheat GRF4 and its cofactor GIF1 significantly increases the efficiency and speed of regeneration in wheat, rice and citrus. Furthermore, it increases the number of transformable wheat genotypes, suggesting potential applications in biotechnology.
Results Unforeseen in the Original Project:
The current paradigm in miRNA biogenesis involved the recognition of the secondary structure by DCL1. We unexpectedly found that there are key aspects of their primary sequence that are essential for their processing.
Publications:
Baulies, J.L., Bresso, E.G., Goldy, C., Palatnik, J.F., Schommer C. Potent inhibition of TCP transcription factors by miR319 ensures proper root growth in Arabidopsis. 2022. Plant Mol. Biol. doi: 10.1007/s11103-021-01227-8

Beltramino, M., Debernardi, J.M., Ferela, A., Palatnik, J.F. ARF2 represses expression of plant GRF transcription factors in a complementary mechanism to microRNA miR396. 2021. Plant Physiol. **185**, 1798-1812

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Liebsch, D., Palatnik, J.F. MicroRNA miR396, GRF transcription factors and GIF co-regulators: a conserved plant growth regulatory module with potential for breeding and biotechnology. 2020. Curr. Opin, Plant, Biol, **53**, 31-42

Rojas, A.M.L., Drusin, S.I., Chorostecki, U., Mateos, J.L., Moro, B., Bologna, N.G., Bresso, E.G., Schapire, A., Rasia, R.M., Moreno, D.M., Palatnik, J.F. Identification of key sequence features required for microRNA biogenesis in plants. 2020. Nat. Commun. **11**, 5320

Manavella, P.A., Yang, S.W., Palatnik, J. Keep calm and carry on: miRNA biogenesis under stress. 2019. Plant J. **99**, 832-843

Moro, B., Chorostecki, U., Arikit, S., Suarez, I.P., Hobartner, C., Rasia, R.M., Meyers, B.C., Palatnik, J.F. Efficiency and precision of microRNA biogenesis modes in plants. 2018. Nucleic Acids Res. **46**, 10709-10723

BOSNIA AND HERZEGOVINA

Title: Understanding the incomplete extravillous trophoblast invasion in pregnancies complicated with severe and non-severe preeclampsia

Principal Investigator: Violeta Šoljić, Laboratory of Morphology, Department of Histology and Embryology, School of Medicine, University of Mostar, Kralja Petra Krešimira IV bb, 88000 Mostar, Bosnia and Herzegovina. Tel:/Fax: +387-36-336600, E-mail: violeta.soljic@mef.sum.ba

ICGEB Contract No.: CRP/19/002

ICGEB Reference No.: CRP/BIH18-03

Abstract: Preeclampsia (PE) is considered to be the most common cause of death in foetal and perinatal period but its etiology remains unknown. The subjects of research are "cancer-like" mechanisms affecting the invasion and differentiation of extravillous trophoblast (EVT) during the development of the placenta, that, in the case of deregulation, may lead to development of PE. Previously, it is suggested that low IMP3 expression leads to a shallow invasion of EVT, incomplete remodelling of spiral arteries, and consequently to the development of PE. Another crucial aspect that may play role in the development of PE is maternal immunological response occurring in peripheral blood and in placenta. We found significantly decreased expression of IMP3 and LIN28A (onco-fetal proteins) in EVT cells. CD8+T cells from women with PE showed decreased expression of cytotoxic proteins in peripheral blood and in decidua basalis obtained from placenta. Majority of decidual CD8+T cells were naïve and effector in PE, while in the decidua basalis of the control group effector and effector memory were most abundant. Severe PE is characterised by altered expression of cytotoxic CD8+ T cells in decidua and peripheral blood, suggesting their role in pathophysiology of PE and foetal-maternal immune tolerance.

Publications:

Orlovic Vlaho, M., Tomic, V., Vukojevic, K. et al. CD25+FOXP3+ and CD4+CD25+ cells distribution in decidual departments of women with severe and mild pre-eclampsia: Comparison with healthy pregnancies. 2020. Am. J. Reprod. Immunol. **84**, e13281

Soljic, V., Barbaric, M., Vukoja, M., Curlin, M., Orlovic Vlaho, M., CerniObrdalj, E., Lasic Arapovic, L., Bevanda Glibo, D., Vukojevic, K. Decreased expression of cytotoxic proteins in decidual CD8+ T cells in preeclampsia. 2021. Biology (Basel), **10(10)**, 1037

BRAZIL

Title: One Health International Network for SARS-CoV-2 real time genomic monitoring
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ICGEB Contract No.: CRP/21/011
ICGEB Reference No.: CRP/BRA20-03
Project funded by IILA
Abstract: Understanding the spread of viral infectious diseases is crucial for informing public health decisions. This is the case of SARS-CoV-2 pandemic, which presents a global challenge and demands a rapid sanitary response. The present proposal aimed the establishment of a One Health International Network between Oswaldo Cruz Foundation in Brazil and the Laboratory of Molecular Virology of the International Centre for Genetic Engineering and Biotechnology (ICGEB) in Italy for the real-time SARS-CoV-2 genomic monitoring, for a rapid massive viral genome sequencing and large-scale analysis. Through this network we studied the dynamics and the evolution of this emerging virus by monitoring viral transmission, both in the current outbreak and in the post-pandemic period, in preparation for subsequent epidemic waves. The obtained knowledge helped to elucidate important aspects of clinical/epidemiological relevance, which are keys for the design/tailoring of control/preventive strategies along COVID-19 pandemic supporting the evaluation of drugs, vaccines and non-pharmacological interventions.

Objectives:

Our main goal was to establish a One Health International Network for rapid massive SARS-CoV-2 genomic sequencing, monitoring, and large-scale analysis.

Results Obtained:

During the 12-month period of execution of the present proposal we were fully involved in the establishment of an International Network between Brazil and Italy for the real- time genomic monitoring to foster generation of knowledge about SARS-CoV-2, with an emphasis on real-time sequencing, which allowed addressing issues of epidemiological, virological, and clinical relevance. During the execution of this project, we generated over 10,000 SARS-CoV-2 complete genome sequences (most of them were from Brazil) using both nanopore as well as Illumina MiSeq technologies. Generated data were (and will be) subjected to open release as they were generated (GISAID; virological.org; Twitter). The analyses of the partial and final results were made available through technical reports to the Italian, and the Brazilian Ministry of Health and via published articles in indexed scientific journals. During this time 10 research articles have been produced which 7 of them have been already published in international scientific journals and 3 of them are still in a peer-review process. Additionally, students from several Brazilian universities including the Federal University of Minas Gerais and Rio de Janeiro have been trained to provide hands-on genomics surveillance. Training programs included dry and wet-lab webinars and face-to-face activities in which students were trained to understand the entire workflow. Those activities included: (i) molecular screening (using Real-time PCR assays); (ii) NGS technology for the generation of new SARS-CoV-2 complete genome sequences; (iii) analysis of NGS raw data; (iv) phylogenetic and phylodynamic inferences and v) integration of genomic data with epidemiological and mobility ones. Importantly, the epidemiological and genomic data analysed in real-time during the training program and subsequently during online sessions, and the participants attending the training program made a significant contribution to the research outputs. These initiatives have the potential to build local capacity in the field of genomic surveillance and to advance our understanding on the population biology of circulating viruses and other emerging pathogens.

Studies involving more in-depth molecular analyses and dispersion of circulating infectious pathogens can help the Brazilian and Italian Ministries of Health to adopt appropriate measures to control epidemics/pandemics and monitor the dynamics and spread of new viruses. This proposal will further contribute to the application and consolidation of state-of-the-art sequencing and computational tools in the research of emerging and re-emerging viral pathogens at the international level, thus contributing to the resolution of issues relevant to Public Health while advancing technological innovation in Biological, Biotechnology and Health Sciences.

Publications:

Broccolo, F., Giovanetti, M., Colombo, A., et al. Molecular detection of SARS-CoV-2 eta VOI in Northern Italy: A case report. 2021. Clin. Chem. Lab. Med. 60(3), 61-63

Giovanetti, M., Cella, E., Benedetti, F., et al. SARS-CoV-2 shifting transmission dynamics and hidden reservoirs potentially limit efficacy of public health interventions in Italy. 2021. Commun. Biol. 4(1), 489

Giovanetti, M., et al. Replacement of the Gamma by the Delta variant in Brazil: Impact of lineage displacement on the ongoing pandemic. 2021. MedRxiv. doi.org/10.1101/2021.12.27.21268309

Angeletti, S., Giovanetti, M., Fogolari, M., et al. SARS-CoV-2 AY.4.2 variant circulating in Italy: Genomic preliminary insight. 2022. J. Med. Virol. 94(4), 1689-1692
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Maruotti, A., Giovanetti, M., Divino, F., Broccolo, F., Angeletti, S., Ciccozzi, M. Prisoners of variants, or free to act as prisoners of swabs? The case of Italy. 2022. J. Med. Virol. **94(6)**, 2334-2335
Pascarella, S., Bianchi, M., Giovanetti, M., et al. The SARS-CoV-2 Mu variant should not be left aside: It warrants attention for its immuno-escaping ability. 2022. J. Med. Virol. **94(6)**,2479-2486
Alcantara, L.C.J., Nogueira, E., Shuab, G., Tosta, S., Fristch, H., Pimentel, V., Souza-Neto, J.A., Lehmann Coutinho, L., Fukumasu, H., Coccuzzo Sampaio, S., Elias, M.C., Kashima, S., Nanev Slavov, S., Ciccozzi, M., Cella, E., Lourenco, J., Fonseca, V., Giovanetti, M. SARS-CoV-2 epidemic in Brazil: how variants displacement have driven distinct epidemic waves. 2022. Virus Evol. (accepted)

CHILE

Title: Targeting type-I and type-II natural killer T cells to enhance effector and memory B cell responses

Principal Investigator: Leandro J. Carreño, Millennium Institute on Immunology and Immunotherapy, Programa de Inmunología, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Av. Independencia 1027, Block I, Piso 3, Santiago, Chile

ICGEB Contract No.: CRP/18/005

ICGEB Reference No.: CRP/CHL17-06_EC

Abstract: Natural Killer T (NKT) cells are a specialised group of unconventional T cells, characterised by the co- expression of α β-T cell receptors (TCRs) together with multiple receptors that are typically associated with natural killer (NK) cells, which gives them their name. These cells can modulate the outcome of immune responses against pathogens, tumours, allergens and self-antigens, and thus are important targets for potential immunotherapeutic strategies. Since these cells can be differentially activated by different glycolipids bound to the CD1d molecule, inducing different cytokine signatures, in this project, we evaluated whether the activation of NKT cells by different ligands can modulate the response of B cells, impacting antibody secretion and memory. By using liposomes containing B cell antigens and different glycolipid that activate NKT cells, we evaluate the magnitude of antibody secretion, the specific isotypes produced, their affinity, and the establishment of B cell memory. Our results shown that NKT cell activation can modulate B cell responses depending on the characteristics of the NKT cell activation, enhancing antibody production, affinity and memory, and also fine-tunning isotype switching.

Objectives:

(i) To generate NKT- and B cell -stimulating liposomes for concomitant delivery of B cell-antigens and NKT cell activating lipid-ligands;

(ii) To evaluate the magnitude and isotypes of antigen-specific antibodies after concomitant stimulation of B cells and NKT cells;

(iii) To evaluate the generation and establishment of memory B cells after concomitant stimulation of B cells and NKT cells.

Results Obtained:

The results of this project shown that concomitant NKT cell and B cell activation results in a significant increase of antibody production, an increase on antibody affinity and a significant enhancement on the establishment on long-lived plasma cells that produce antibodies, indicative of an increase on B cell memory. Interestingly, depending on the nature of NKT cell activation, there is a fine tunning of the isotype switching, being the isotypes IgG1 and IgG2c more prevalent when NKT cells are activated with pro- inflammatory ligands.

Results Unforeseen in the Original Project:

In addition to the results obtained in this project, during our working with liposomes containing antigens and NKT cell ligands we identify anti-inflammatory NKT cell ligands that induce the expansion of a tolerogenic population of NKT cells, known as NKT10.

COSTA RICA

Title: Development and validation of a genetically encoded fluorescent reporter for Dengue and Zika Virus NS2B-NS3 protease activity and its application to explore the phenotypic diversity of viral infection in terms of replication, cell death induction and antiviral drug response

Principal Investigator: Rodrigo Mora Rodríguez, Centro de Investigación en Enfermedades Tropicales (CIET), Universidad de Costa Rica, Ciudad Universitaria Rodrigo Facio, San Pedro de Montes de Oca, San José, 11501-2060, Costa Rica. Tel: +506-25118635, E-mail: rodrigo.morarodriguez@ucr.ac.cr

ICGEB Contract No.: CRP/19/004
ICGEB Reference No.: CRP/CRI18-02/A

Abstract: The quest for therapeutic targets to combat flavivirus infections requires a better understanding of the kinetics of the virus-cell interplay, which is hindered by limitations of the current cell-based systems for monitoring flavivirus infection by live-cell imaging. The present project undertook the development and validation of fluorescence-activatable sensors to detect the activity of flavivirus NS2B-NS3 serine proteases in living cells. A GFP version of this sensor containing the flavivirus internal NS3 cleavage site linker (AAQRRGRIG) reported the highest fluorescence activation in stably transduced mammalian cells upon DENV-2/ZIKV infection. Moreover, a far-red version of this flavivirus sensor presented the best signal-to-noise ratio in a fluorescent Dulbecco's plaque assay, leading to the construction of a multireporter platform combining the flavivirus sensor with DNA fluorescent dyes for the detection of virus-induced chromatin condensation and cell death to study of kinetics of infection and cytophatic effect induction by DENV-2, ZIKV, and YFV in cell-subpopulations and the screening of antiviral drugs to manage flavivirus infections.

Objectives:

(i) To develop a cellular system stably-expressing a genetically encoded reporter tested in a cell-free system to become fluorescent upon NS3 protease cleavage *in vitro*;
(ii) To evaluate the cellular system stably-expressing the genetically encoded reporter of NS3 activity upon a cellular infection with Dengue and Zika virions;
(iii) To validate the biological function of the cellular system stably-expressing the genetically encoded reporter of NS3 activity upon a cellular infection with Dengue and Zika virions;
(iv) To explore the range of dynamic cellular activities of several flaviviruses in terms of viral replication and cell death;
(v) To evaluate the potential of this system for antiviral drug developing by characterising the activities of novel antiviral compounds on the dynamics of viral replication and cell death.

Results Obtained:

In the present study, we report the construction of fluorescence-activatable sensors to detect the activities of flavivirus NS2B–NS3 serine proteases in living cells. The system consists of GFP-based reporters that become fluorescent upon cleavage by recombinant DENV-2/ZIKV proteases *in vitro*. A version of this sensor containing the flavivirus internal NS3 cleavage site linker reported the highest fluorescence activation in stably transduced mammalian cells upon DENV-2/ZIKV infection. Moreover, the onset of fluorescence correlated with viral protease activity. A far-red version of this flavivirus sensor had the best signal-to-noise ratio in a fluorescent Dulbecco's plaque assay, leading to the construction of a multireporter platform combining the flavivirus sensor with reporter dyes for detection of chromatin condensation and cell death, enabling studies of viral plaque formation with single-cell resolution. The application of this platform enabled the study of cell-population kinetics of infection and cell death by DENV-2, ZIKV, and yellow fever virus. These results are published in https://doi.org/10.1074/jbc.RA119.011319.

We compared several methods in their capacity to evaluate antiviral activities. Conventional plaque assays rely on the use of overlays to restrict viral infection allowing the formation of distinct foci that grow in time as the replication cycle continues leading to countable plaques that are visualised with standard techniques such as crystal violet, neutral red, or immunolabeling. This classical approach takes several days until large enough plaques can be visualised and counted with some variation due to subjectivity in plaque recognition. Since plaques are clonal lesions produced by virus-induced cytopathic effect, we applied DNA fluorescent dyes with differential cell permeability to visualise them by live-cell imaging. We could observe different stages of that cytopathic effect corresponding to an early wave of cells with chromatin-condensation followed by a wave of dead cells with membrane permeabilization within plaques generated by different animal viruses. This approach enables an automated plaque identification using image analysis to increase single plaque resolution compared to crystal violet counterstaining and allows its application to plaque tracking and plaque reduction assays to test compounds for both antiviral and cytotoxic activities. Since we could not obtain or replicate a DENV replicon, we did these studies with a HSV-1 virus expressing green fluorescent protein. These results were published in https://www.mdpi.com/1999-4915/13/7/1193.

Lastly, we conducted a compound docking approach against the structures of the DENV and ZIKV protease (see methodology) to prioritise the search of antiviral compounds to be tested. The low free

binding energies indicate an adequate affinity of the respective compounds to the protease to be considered effective. Interestingly, this binding involves residues of the catalytic triad of the NS3/NS2B protease, corresponding to the active site of the enzyme. In addition, the pharmacological analysis of the physico-chemical properties predicts good pharmacokinetic properties of the five selected compounds.

Our positive control of NS3 inhibition was effective blocking both the activation of the FlaviA-GFP and the virus-induced cytophatic effect as previously reported (Cell Research 2017 27:1046-1064). None of the four compounds tested showed a complete antiviral effect, as evidenced by both activation of the FlaviA-GFP and a cytophatic effect similar to the mock-treated control. Only the compound AMB6002 showed a slightly antiviral activity evidenced by a reduction in the number of dead cells, nevertheless, the doses tested for all the compounds were the higher doses that were no cytotoxic to the cellular model and constitute the limit of what could be screened for antiviral effect.

Results Unforeseen in the Original Project:

Moreover, we optimised all our protocols with details and documented this in the shape of a manuscript for the journal Bio-protocol (https://bio-protocol.org/Default.aspx), that was published in 2021: https://bio-protocol.org/e3942. There we provided a detailed protocol for the generation, selection and implementation of stable BHK-21 cells expressing our flavivirus genetically-encoded molecular reporters, suitable to monitor the viral infection by live-cell imaging. We also described the image analysis procedures and provide the required software pipelines. Our reporter cells allow the implementation of single-cell infection kinetics as well as plaque assays for both reference and native strains of flaviviruses by live-cell imaging. In addition, we wrote a book chapter for Springer where we discussed the basics of the fluorescence imaging techniques currently employed in flavivirus research, including immunofluorescence assay (IFA), fluorescence in situ hybridisation (FISH), fluorescence-labelled viral particles, fluorescent labelling of cytopathic effect (CPE), subgenomic reporter replicons (SRRs)/reporter virus particles (RVPs), and cell-based molecular reporters (CBMRs). We also addressed the advantages of each application based on our own experience and included some of our protocols to facilitate its applications: https://link.springer.com/chapter/10.1007%2F978-3-030-71165-8_34

Publications:

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HUNGARY

Title: Broad and efficient gene expression via systemic gene delivery of engineered AAVs in a preclinical species

Principal Investigator: Daniel Hillier, Visual Systems Neuroscience Laboratory, Institute of Cognitive Neuroscience and Psychology, Research Centre for Natural Sciences, Magyar Tudosok Korutja 2, 1117 Budapest, Hungary. E-mail: hillier.daniel@ttk.hu

ICGEB Contract No.: CRP/20/022

ICGEB Reference No.: CRP/HUN20-01

Abstract: The goal of the project is to yield a safe, long-term stable method for genetically targeted functional access across the brain in large-animal preclinical species. Current practice is to deliver the AAV via invasive local injections that can accommodate short-term research needs but invasive delivery and immune reaction to vector and transgene make chronic applications difficult and preclude translation to human therapeutic purposes. Our aim is to identify AAV capsid and promoter combinations that can yield strong expression upon non-invasive delivery route in targeted neurons. Demonstration of stable, long-term functional access to neuronal function upon non-invasive gene delivery may be essential both for basic scientists aiming to dissect neuronal function in preclinical species and for therapy of brain disorders.

Objectives:

The field of large animal neuroscience would benefit very much from the establishment of efficient and stable methods for genetically targeted gene delivery to the brain. Identification of a gene delivery method that yields brain-wide, long-term stable and functional labelling may surmount a major, long-standing methodological barrier that may enable the field to realize a whole new set of experiments. Calcium imaging and optogenetics have been demonstrated upon local AAV delivery. However, variability and unpredictability of labelling efficiency remain as major hurdles. Local delivery is invasive, induces immune response and potentially causes structural remodelling. The regulatory and financial burden of cat experiments is significantly lower than that of primates. Beyond the practical and financial aspects, Directive 2010/63/EU also mandates the use of non-primate species for research where primates are not absolutely necessary. The example of a recent failed macaque AAV delivery study warrants caution and strongly supports establishment and calibration of molecular tools in lower species. The extent and rules on cross-species translatability of molecular is not well understood and in-bred mouse strains may not be the best indicator about translation potential of an AAV serotype. In contrast, large preclinical species are not inbred have potentially much higher translational value for human applicability. Indeed, once our experiments achieve functional brain-wide labelling, validation of our method in primates will be the next step. These reasons motivated us to invest into a large animal species, cats, and prepare for application in non-human primates after successful demonstration of a working brain-wide labelling strategy in cats.

Results obtained:

To test whether efficient AAV serotypes that showed cross-species generalised tropism also infect cat brain tissue, we performed serial injections in cat visual cortex and detected high expression with the latest set of serotypes that have been shown to yield brain-wide expression in mice and marmosets. After observing the strong labelling of serotype candidates, we proceeded to find the safe viral dose to be injected for whole brain labelling. Our first milestone has been the identification of a safe injection dose that yields brain-wide transgene expression that would allow functional, genetically targeted circuit activity modulation. We obtained a negative result 6 months upon systemic injection of PHP.eB into a young kitten. After observing no transgene expression (by immunohistochemistry and viral DNA quantification), we argued that in addition to increasing the dose, constraining biodistribution of the AAV may lead to faster identification of a working protocol. Thus, we tested intrathecal delivery route that requires less virus to perfuse the brain. Using the same dose as we used for intravenous injection, we injected an adult cat via intra cisterna magna route. This experiment yielded detectable, but still very few transgene expressing cells across the brain. Thus, we increased the dose that yielded very clear labelling with inhomogeneous distribution across the cortex and almost general lack of labelling in subcortical regions. We cloned and produced new AAV constructs that were successfully used for local AAV delivery in macaques. We have built a wide-field fluorescence microscope and validated it with recording visually-evoked activity from the visual cortex of rats. In summary, we could identify a brain-wide AAV delivery strategy, developed new constructs for increasing transgene expression levels, established a new, stable anaesthesia protocol for recording brain activity from cats, established functional fluorescence imaging in a lower species. Via intrathecal delivery, new amplification constructs and immunomodulation strategies may increase expression levels and homogeneity. These results may provide a solid ground for subsequent validation in non-human primates.

KENYA

Title: Generating blast resistant finger millet plants through gene-specific genome editing
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ICGEB Contract No.: CRP/CRP/19/006
ICGEB Reference No.: CRP/KEN18-01
Abstract: Blast disease, caused by *Magnaporthe oryzae*, is the most devastating disease affecting finger millet, a staple food for millions of people in the semi-arid tropics of East Africa. The fungus infects the crop at all stages of growth and most of the land-races. The disease causes failure of the grain to set and seeds to shrivel, resulting to up to 90% yield losses. The adoption of host resistance has proven to be the most economical and effective approach to control finger millet blast disease. CRISPR/Cas genome editing technology has transformed plant biotechnology beyond all expectations. CRISPR/Cas system allows precise genetic manipulation of plants, providing opportunity to create germplasms with beneficial traits. Not a single report on finger millet trait improvement by using the CRISPR/Cas system is available. This project was designed to enhance finger millet blast resistance by CRISPR/Cas9-targeted mutagenesis of the plant ethylene response factor transcription factor gene, which is involved in the modulation of multiple stress tolerance. The CRISPR/Cas9 system for finger millet blast resistance developed paves way for large-scale RNA-guided genome editing in finger millet to enhance its breeding and the crop's research. Mitigating the effects of *M. oryzae* on finger millet, will allow the crop to play a significant role in the fight against hunger and malnutrition.
Objectives:
(i) Establish an efficient CRISPR/Cas9 genome in finger millet by targeting Phytoene desaturase gene;
(ii) Enhance finger millet blast resistance by CRISPR/Cas9-targeted mutagenesis of the plant ethylene response factor transcription factor gene in finger millet varieties widely cultivated in Kenya.
Results Obtained:
The project was established an efficient CRISPR/Cas9-mediated targeting of Phytoene desaturase (PDS) in five finger millet varieties. These results set the groundwork for future studies of gene function and trait development using the CRISPR/Cas9 gene-editing technology in finger millet. The project also obtained genome edited finger millet plants that harbour mutations in the ethylene response factor transcription factor gene. Inoculation with *M. oryzae* revealed enhanced blast resistance in the mutant lines when compared to of wild-type plants at both the seedling stages. In addition, there was no significant difference between homozygous mutant lines and wild-type plants with respect to the agronomic traits. This work offers an effective process of enhancing finger millet blast resistance using CRISPR/Cas9 technology.
Publications:
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Juma, B.S., Mukami, A., Mweu, C., Ngugi, M.P., Mbinda, W. Targeted mutagenesis of the *CYP79D1* gene *via* CRISPR/Cas9-mediated genome editing results in lower levels of cyanide in cassava. 2022. Front. Plant Sci. **13**, 1009860

MALAYSIA

Title: Vagus nerve stimulation: Investigation on cholinergic anti-inflammatory activity and macrophage polarization in rats with induced myocardial infarction

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ICGEB Contract No.: CRP/19/007

ICGEB Reference No.: CRP/MYS18-04_EC

Abstract: An imbalance between pro- and anti-inflammatory transitions in post myocardial infarction (MI) may result in cardiac dysfunction. The purpose of this study is to investigate the cardioprotective mechanism of transcutaneous vagus nerve stimulation (TVNS) by investigating the cholinergic anti-inflammatory pathway (CAP) and macrophage polarisation. MI was induced with a two-day subcutaneous injection of isoprenaline hydrochloride (85mg/kg). Electrical stimulation at tragus with stimulation intensity of 20Hz, 0.2ms, 2mA, 1 hour daily for 14 days was used to stimulate the vagus nerve. The Langendorff apparatus was used to evaluate left ventricular function. Left ventricular developed pressure (LVDP), maximum rate of left ventricular pressure increase (LV +dp/dtmax), maximum rate of left ventricular pressure decrease during relaxation (LV -dp/dtmax), and heart rate all improved. Vagal activation reduced cellular hypertrophy and fibrosis, as measured by H&E and Picrosirius staining, respectively. The flow cytometric analysis revealed that the vagal stimulated group had a higher percentage of M2 (CD11b+F4/80+CD206+). Nonetheless, in sham stimulation or cholinergic antagonist groups, the positive effects were reduced. The findings of this study show that TVNS has the potential to improve ventricular function, inflammation, fibrosis, and ventricular remodelling, as well as potentially induce macrophage polarisation towards M2 phenotype.

Objectives:

This study aims to examine the cardioprotective mechanism of transcutaneous vagus nerve stimulation (TVNS) by looking into cholinergic anti-inflammatory pathway (CAP) and macrophages polarisation. Our research project is being outlined based on these specific objectives:

(i) To examine cardioprotective effects of auricular vagus nerve stimulation;

(ii) To examine nicotinic pathway from auricular vagus nerve stimulation;

(iii) To examine cardiac macrophages polarisation in response to vagus stimulation.

Results Obtained:

The effects of tVNS on cardioprotection were studied using a Langerdorff isolated heart preparation and immunohistology. The MI+TVNS group had a significant increase in LVDP when compared to the MI group. Similarly, the MI +TVNS group had a significant increase in LV +dp/dtmax values ($p < 0.05$) when compared to the MI group. Heart rate was significantly lower in the MI+tVNS group. H&E staining showed the MI+TVNS group had a smaller cardiomyocyte size. The IM+TVNS group showed a trend of reduction in the area of collagen deposition by the PicoSirius staining. In sham stimulation animals, these cardioprotective effects were suppressed.

We then compared TVNS to groups that had been treated with a nicotinic receptor antagonist to see if auricular vagus nerve stimulation activated the nicotinic anti-inflammatory pathway. In MI animals, the TVNS treatment showed a trend of decreased circulating TNF. This trend was reversed in the sham stimulation group or in animals with nicotinic receptor blockade. The differences in blood IL-1 concentrations between all groups were found to be non-statistically significant.

Following that, we investigated macrophage polarisation in response to vagus stimulation. First, we looked at F4/80+ CD11b+ cardiac macrophages in both healthy and MI groups. When compared to the healthy group, the MI group (45.21%) showed a pattern of reduction in the CD11b+ F4/80+ stained population (71.31%), indicating a significant drop in total macrophage count. Furthermore, the percentage of CD206 receptor expression was compared to determine the phenotype of macrophages. Our findings revealed that macrophages from healthy animals (69.33%) and MI (47.83%) did not exhibit the pro-inflammatory phenotype (CD11b+F4/80+CD206-). However, macrophages from both groups exhibited an anti-inflammatory phenotype (CD11b+F4/80+CD206+), with 99.55 percent of healthy macrophages and 99.93 percent of IM macrophages expressing CD206 receptors. The effects of TVNS on macrophages expression in MI rats were examined. The MI+TVNS group (64.37 %) showed a pattern of increasing percentage of CD11b+ F4/80+ macrophage when compared to the IM group (45.53 %). Nevertheless, the percentage of the CD11b+ F4/80+ macrophage was reverted upon sham stimulation (52.49 %). A similar pattern was also observed in the IM+TVNS+Atropine (51.30 %) and IM+TVNS+α-Bungarotoxin (52.40 %) groups.

Results Unforeseen in the Original Project:

Examination on the expression of proinflammatory macrophages CD11b+F4/80+CD206- in the MI hearts of MI, MI+TVNS, MI+SS, IM+TVNS+Atropine and IM+TVNS+α Bungarotoxin was non-reactive. However, macrophages from the group expressed the anti-inflammatory phenotype M2 (CD11b+F4/80+CD206+) in which 99.93 % macrophages from the IM group, 100.0 % macrophages

from the IM+TVNS group, 98.91 % macrophages from the IM+SS group, 99.92 % macrophages from the IM+TVNS+A group and 99.89 % of macrophages from the IM+TVNS+A group expressed CD206 receptors.

Publications:

Chung, C.H., Bretherton, B., Zainalabidin, S., Deuchars, S.A., Deuchars, J., Mahadi, M.K. Mediation of cardiac macrophage activity via auricular vagal nerve stimulation ameliorates cardiac ischemia/reperfusion injury. 2020. Front. Neurosci. **14**, 906

MEXICO

Title: Human germinal centre organoids as a tool to understand B cell biology and for the identification of monoclonal antibodies with therapeutic potential against SARS-CoV2

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ICGEB Contract No.: CRP/20/012

ICGEB Reference No.: CRP/MEX20-04_EC

Abstract: High-affinity antibodies (Abs) required for pathogen neutralisation and vaccine efficacy are usually produced in sub-anatomical regions known as germinal centres (GC). High-affinity/avidity B cell receptor (BCR) interaction with the antigen is a key-determining factor in the efficiency of the GC reaction. We hypothesised that the application of antigen-specific clonal selection methods in a 3D culture of human B cells would allow the selective activation and expansion of B cells, and the generation of human high-affinity Abs against SARS-CoV2. We developed a specific targeting and delivery system by loading antigen-coated chitosan nanoparticles (Ch-Np) with co-stimulatory molecules to introduce them into specific B cells. We characterised the Ch-Np, and tested the best microenvironmental conditions to stimulate B cells isolated from convalescent COVID-19 patients. The stimulation with the multimerised antigen induced more cells secreting antigen-specific Abs, whereas the presence of Bcl-6 in the Ch-Np increases cellular viability. Our stimulation protocol, applied in a 3D or 2D culture, induced B cell differentiation expanding the proportion of plasma and memory B cells.

Objectives:

Our aims were: (i) to generate antigen-specific selection methods that yield antigen-specific mAbs with higher signal/noise ratios using nanotechnological approaches and (ii) to determine the ideal conditions to mimic the GC reaction *in vitro*.

Results Obtained:

We prepared and characterised antigen-bound chitosan nanoparticles (Ch-Np) that encapsulate a vector encoding the Bcl-6 protein or with CpG oligodeoxynucleotides. Since Bcl-6 is a fundamental regulator of the GC reaction, we prepared Ch-Np that incorporate a vector coding for this protein. Bcl-6 amplification was performed from a human amygdala sample by purifying the mRNA and performing cDNA synthesis and PCR in one step, then it was cloned in the plasmid pIRES2-EGFP. Sequencing was performed to verify the correct orientation of the Bcl-6 gene. We transfected the Bcl-6 construct into HEK-293T cells and evaluated EGFP expression under a fluorescence microscope to indirectly corroborate the expression of Bcl-6. Then, we generated Ch-Np containing the vector coding for Bcl-6 or the empty vector, and conjugated with a protein (antigen). We evaluated and determined the efficiency of different protocols for protein binding to Ch-Np. For standardisation studies, we prepared bovine serum albumin (BSA)-bound Ch-Np, whereas Staphylococcus aureus protein A (SpA)-bound Ch-Np were used as controls to evaluate incorporation specificity. As antigen of interest, we used the recombinant receptor binding domain (RBD) of the S1 subunit from SARS-CoV-2. Protein binding efficiency was evaluated in the supernatants generated during the preparation of the BSA-bound Ch-NP by bicinchoninic acid assays and non-bound protein was visualised by SDS-Page. The incorporation of the DNA was evaluated by electrophoresis in agarose gels and by spectroscopy at 260 nm. We characterised the shape, size, and charge of all the preparations generated by transmission electron microscopy and tunable resistive pulse sensing. Despite their content, the nanoparticles had a uniformly spherical shape with average diameters between 99 and 224 nm and were negatively charged.

To test the specificity for the antigen, 2 cell lines of B lymphocytes were incubated with Ch-Np coated with SpA, Daudi cells that belong to clan III of the immunoglobulins and that recognise the antigen; and Ramos cells, belonging to clan II and which were our negative control. We used BSA-Ch-Np to standardise the stimulation conditions, several Ch-Np concentrations and incubation times were tested and evaluated by a proliferation assay using MTS/PMS. As our purpose is to apply this model for the identification of mAbs against SARS-CoV-2, we used the RBD-Ch-Np to stimulate *in vitro* B cells derived from COVID-19-recovered volunteers. We recruited 50 volunteers including COVID-19-recovered patients or SARS-CoV-2 vaccinated individuals for blood collection. We analysed their RBD-antibody status by ELISA and selected 5 with the highest RBD-antibodies titers for B cell isolation. Anti-RBD Abs titers among the selected volunteers were around 1:1000 to 1:4000. Using FITC-conjugated nanoparticles, we determined the rate of incorporation into B cells at different times and concentrations by flow cytometry. We corroborated the incorporation of the RBD-Ch-Np into B cells by confocal microscopy.

We tested the best microenvironmental conditions to stimulate B cells. We stimulated B cells *in vitro* using protocols (cytokines cocktails) described by other research groups and some generated from our own experience. In some of the protocols, we included non-B cells which correspond to the mononuclear cells obtained during B cell separation. Stimulation with the cytokines but without

antigen was also tested, whereas no stimulated cells were used as a negative control. We detected the production of IgG antibodies in all the stimulated cultures; however, the generation of specific antibodies was higher using one of our designed protocols. Moreover, the presence of specific antibody-secreting cells was more evident using this protocol on day 7 post-stimulation as determined by ELISPOT. Then, we stimulated B cells using the most successful protocol and compared between the conventional 2D culture and 3D culture using alginate beads and Matrigel as scaffolding materials. B cell viability was higher during the first days of culture in the 3D model, whereas as total IgG production is similar in the 2D and the 3D models during all the longitudinal analyses. Interestingly, specific antibody production is more robust in the 3D model.

In conclusion, we found that stimulation with the multimerised antigen in nanoparticles induced more cells secreting antigen-specific antibodies and, subsequently, more specific anti-RBD antibodies than the soluble antigen. Furthermore, the presence of Bcl-6 contained in the nanoparticles increases the viability of the stimulated cells. The stimulation protocol we designed, applied in a 3D or 2D culture, induced B cell differentiation, reducing the number of naive B cells and increasing the proportion of memory B cells and plasma cells. We will continue to study this model to determine the factors necessary to increase the proportion of GC B cells and their maintenance *in vitro*.

Results Unforeseen in the Original Project:

ICGEB funding has contributed significantly to the scientific training of 2 MSc students and 1 undergraduate student who completed their theses under this project. A Ph.D. student recently joined the project and received training under this grant. One of the master's students submitted a review article (in press); the other had the opportunity to be trained in pioneering tissue engineering techniques to apply to the project (financed by ICGEB) and prepared a manuscript for publication derived from her thesis project.

RUSSIA

Title: QM/MM Approach for remodelling of biocatalytic specificity
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ICGEB Contract No.: CRP/19/008
ICGEB Reference No.: CRP/RUS18-01
Abstract: In final stage of the work we used the developed on the previous stages methodology of QM/MM calculation to direct evolution of enxyme and abzyme active centre to hydrolyse of organophosphorus toxins. A detailed analysis of the QM/MM experiments showed that the histidine residues introduced into the acyl-binding loop and which are crucial for the reactivation of mutant BChE are always located in close proximity to the aspartate residue at position 70. This combination resembles the canonical pair of histidine and carboxylic acid. acidic residues are found in the active site of various proteases. The carboxyl group acts as an activator of the histidine residue, changing its pKa, which in turn contributes to the activation of the water molecule in terms of its nucleophilicity. The observed reprotonation of the catalytic serine residue at position 198 due to proton transfer from the histidine residue at position 438 restores the initial configuration of the active site of the enzyme, which becomes ready for the next catalytic cycle. Therefore, we suggest that the use of the uHTS platform in combination with the deconvolution of molecular mechanisms using QM/MM calculations may open up new horizons for the design of catalytic bioacceptors with respect to OP poisons.
Objectives:
The design of novel biocatalysts became an important fundamental and biotech goal of life science. Previously it was a limited number of approaches to improve catalytic efficiency and re-program catalytic specificity. Most of them were realized by application of rational design of mutants based on refined 3D structures. Screening of representative libraries of enzyme's active centres and immunoglobulin's combining sites may give an extraordinary opportunity to generate improved biocatalysts. In order to enhance this approach, we realised the screening of virtual libraries using supercomputer powered by QM/MM simulation of catalytic chemical transformation. We propose to generate de novo catalytic bioscavengers toward organophosphorus compounds with modified specificity based on the butyrylcholinesterase and Ig active cites. We will estimate optimal variants of these templates using QM/MM predictions. We will change the stereospecificity of the catalyst and improve its catalytic properties. The attempt to arrange catalytic re-programming of reaction pathways will be made
Results Obtained:
Screening of representative libraries of enzyme's active centres and immunoglobulin's combining sites may give an extraordinary opportunity to generate improved biocatalysts. In order to enhance this approach, we realised the screening of virtual libraries using supercomputer powered by QM/MM simulation of catalytic chemical transformation. We propose to generate de novo catalytic bioscavengers toward organophosphorus compounds with modified specificity based on the butyrylcholinesterase and Ig active cites. We will estimate optimal variants of these templates using QM/MM predictions. We will change the stereospecificity of the catalyst and improve its catalytic properties. The attempt to arrange catalytic re-programming of reaction pathways will be made.
Publications:
Bobik, T.V., Kostin, N.N., Skryabin, G.A., Tsabai, P.N., Simonova, M.A., Knorre, V.D., Stratienko, O.N., Aleshenko, N.L., Vorobiev, I.I., Khurs, E.N., Mokrushina, Y.A., Smirnov, I.V., Alekhin, A.I., Nikitin, A.E., Gabibov, A.G. COVID-19 in Russia: Clinical and immunological features of the first-wave patients. 2021. Acta Naturae **13(1)**, 102-115

SLOVAKIA

Title: The tryptophan kynurenine pathway- therapeutic strategy for neuroprotection in tauopathies
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ICGEB Contract No.: CRP/19/016
ICGEB Reference No.: CRP/SVK18-01
Abstract: Tauopathies represent a heterogeneous group of neurodegenerative disorders characterised by abnormal deposition of microtubule-associated protein tau into neurofibrillary tangles (NFTs). Previous results showed that the tryptophan/kynurenine pathway is affected in neurodegenerative disorders such as Alzheimer´s disease and other tauopathies. The tryptophan is metabolically converted into a variety of neuroactive substances including kynurenine, 3-hydroxykynurine, 3-hydroxyanthranilic acid, kynurenic acid and quinolinic acid. Kynurenic acid and quinolinic acid both acts on glutamate receptors. One of the viable hypotheses to explain plasma kynurenine decrease is that neurofibrillary pathology affects its transport across the blood-brain barrier (BBB) to serve as a precursor either for neuroprotective kynurenic acid or toxic quinolinic acid. In this project we examined the effect of administration of neuroprotective analog of a kynurenic acid - N-(2-N,N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride (KYNA-1). When chronically administered, KYNA-1 reduced hyperphosporylation of insoluble tau in brain of tau transgenic rats. Noteworthily, the plasma total tau was also reduced. We determined that the effect of KYNA-1 on tau pathology was induced through the modulation of neuroinflammation and astrogliosis.
Objectives:
(i) To describe the role of tryptophan and kynurenine metabolism in the process of tau induced neurodegeneration;
(ii) To describe the relation between neurodegeneration, neuroinflammation and tryptophan metabolism in tauopathies;
(iii) To use synthetic BBB permeable derivate of neuroprotective kynurenic acid N-(2-N,N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamide hydrochloride and describe its effect on neurodegeneration and neuroinflammation in SHR-24 animal model for tauopathies;
(iv) To analyse of L-kynurenine transport across the BBB in SHR-24 animal model for tauopathies.
Results Obtained:
To overcome blood-brain barrier limitations we used kynurenic acid analog with neuroprotective activity but higher brain permeability. In present study we used amide derivate of kynurenic acid N-(2-N, N-dimethylaminoethyl)-4-oxo-1H-quinoline-2-carboxamid (KYNA-1). KYNA-1 showed low cytotoxicity. IC50 value was 16.1 ± 0.9 mM in primary cultures of rat astrocytes and 15.9 ± 1.49 mM in SY5/2 cells. To examine whether the KYNA-1 analog could penetrate across BBB, we used an in vitro BBB model. Primary rat endothelial cells were seeded onto the apical side of a Transwell inserts and co- cultured with primary rat astrocytes for 7 days to encourage the formation of a tight cell monolayer which expresses a tight junction proteins. KYNA-1 analog was added to the luminal part of the BBB model and the concentration of KYNA-1 was quantified using LC-MS/MS method. The results showed that KYNA-1 was able to cross the endothelial monolayer. Its permeability significantly increased in time- dependent manner.. The endothelial permeability coefficient (Pe) of KYNA-1 was calculated 1.1 x 10-7 cm/ s-1. The Pe of KYNA-1 was 5.5 times higher than Pe of kynurenic acid. We calculated the pharmacokinetics parameters of KYNA-1 after single i.p. administration. After the single intraperitoneal dose (200mg.kg-1) the levels of KYNA-1 peaked in serum and brain tissue at 30 minutes and then slowly decreased with a half life of 2.6h. The AUC0-t value was 191507.32 ng/ml*h. The maximum brain levels were approximately 45-53 times lower than in the plasma. The unidirectional influx rate of KYNA-1 in frontal cortex and brainstem was 11.85±1.6 µl/g-min and 7.05±1.9 µl/g-min respectively.
Next we analysed the levels of KP metabolites (kynurenine, kynurenic acid, anthranilic acid and xanthurenic acid) in plasma and brain tissue of injected animals. We found that 30 minutes after administration of KYNA-1 the plasma levels of KA significantly increased in compare to basal levels (6.51±1.32ng/ml at t0 vs 475.4±188.5ng/ml at t30), then returning to basal values after 12 hours. The plasma levels of anthranilic acid gradually increased up to 12h, then slowly returned to basal values at 48 hours. The levels of kynurenine and xanthurenic acid remained unchanged. Interestingly, we observed no change of KA levels in brainstem and frontal cortex at any time point. To determine the effect of KYNA-1 on tau pathology and activation of glial cells we administered KYNA-1 for three months to tau transgenic rats SHR-24. To examine whether KYNA-1 analog affected tau pathology, we analysed total tau in plasma and brain tissue as well as levels of hyper-phosphorylated and aggregated forms of tau protein. Compared with placebo, the expression of soluble tau was not significantly changed in KYNA-1 treated animals. Sarkosyl-insoluble tau protein is the main pathological feature of tauopathies. Therefore, we analysed sarkosyl-insoluble protein extracts from the brainstem of SHR-24 transgenic rats after KYNA-1 treatment. To monitor the level

of phosphorylation of insoluble tau protein, we used specific phospho-tau antibodies against pT181, pS202/pT205, and pT212. The results showed that the amount of sarkosyl-insoluble tau significantly decreased in KYNA-1 treated animals compared to the placebo group. We found significantly lower levels of pS202/pT205 (Placebo: 14 090 ± 2340; KYNA: 7889 ± 3160; n= 8, p< 0.001) and pT181 positive tau (Placebo: 14 886 ± 3056; KYNA: 10289 ± 4209; n= 8, p< 0.038) in KYNA-1 treated rats compare to placebo. There was no change in amount of pT212 positive insoluble tau (placebo: 17 114 ± 1048; KYNA-1: 16178 ± 1224; n= 8, p< 0.098). We further analysed the total tau protein in plasma by supersensitive digital ELISA. Results showed that treatment with KYNA-1 decreased the levels of total tau protein in plasma (placebo: 249.5 ± 52.08; KYNA-1: 109.6 ± 16.72; p= 0.02; n= 8). Neuroinflammation is an important hallmark of tauopathies. Activation of astrocytes closely correlates with neurofibrillary pathology. We quantified the GFAP in KYNA-1 treated animals. Compared to placebo treated group, KYNA-1 was able to modulate astrogliosis and decreased GFAP reactivity (placebo: 7488 ± 690.7; KYNA-1: 4812 ± 746.5; n=8; p= 0.02). To show effect of KYNA-1 on inflammatory processes, we investigated the effect of KYNA-1 on LPS- induced cell toxicity and astrocyte activation. Viability assay showed that LPS was able to induce significant cells death (control cells: 99.78 ± 2.41%; LPS: 161.9 ± 4.18%; p< 0.0001; n= 10). However, when KYNA-1 is added to LPS- stimulated cells toxicity significantly decreased (LPS: 161.9 ± 4.18%; LPS+ KYNA-1: 135.8 ± 7.1%; p= 0.0053; n= 10). Activation of glial cells is accompanied by the production of reactive nitrogen species. We studied the effect of KYNA-1 on nitric oxide (NO) production by astrocytes. The LPS significantly induced NO release (control: 3.2±0.4 uM; LPS: 7.1±2.7 uM; p= 0.0001; n= 12). Addition of KYNA-1 significantly inhibited the NO production by cells (LPS: 7.1±2.7 uM; LPS+KYNA-1: 3.8±0.6 uM). Immunofluorescence analysis of primary rat astrocytes showed stellate morphology in control conditions. Conversely, LPS treated astrocytes exhibited higher GFAP signal and ramified morphology with hypertrophic processes. Consistently, the expression of GFAP measured by western blot analysis increased after LPS treatment (control: 100.6 ± 1.6; LPS: 114.8 ± 1.7; n= 6; p< 0.0001). The pre-treatment with either KYNA-1 (LPS: 114.8 ± 1.7; KYNA+ LPS: 95.2 ± 0.7 n= 6; p< 0.0001) markedly attenuated LPS-mediated upregulation of GFAP expression. Using the primary microglia cultures, we analysed protein markers of the M1 (CD16 and CD32) and M2 (CD163) phenotypes. We found that KYNA-1 increased expression of CD163 marker (control: 297.5 ± 43.34; KYNA-1: 529.6 ± 43.35; n= 7; p= 0.0023). Furthermore, the expression of pro-inflammatory CD16 (control: 4929 ± 485.1; KYNA-1: 3006 ± 345.7; n= 7; p= 0.0073) and CD32 (control: 3035 ± 394.4; KYNA-1: 1872 ± 263; n= 7; p= 0.03) markers was significantly decreased. Altogether the above data suggest that KYNA-1 had the ability to reduce hyperphosporylated aggregated tau in the brain and soluble plasma tau. KYNA-1 has also the potential in reducing the glia activation and stimulates anti-inflammatory, scavenging, and regenerative M2 phenotype in microglia.

Publications:

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Piestansky, J., Barath, P., Majerova, P., Galba, J., Mikus, P., Kovacech, B., Kovac, A. A simple and rapid LC-MS/MS and CE-MS/MS analytical strategy for the determination of therapeutic peptides in modern immunotherapeutics and biopharmaceutics. 2020. J. Pharm. Biomed. Anal. **189**, 113449

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SLOVENIA

Title: Sonogenetics: ultrasound-inducible transcriptional regulation
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ICGEB Contract No.: CRP/19/009
ICGEB Reference No.: CRP/SVN18-01
Abstract: We explored the potential of the ultrasound (US) as a non-invasive remote activator of cells coupled with the expression of the genes of interest. Calcium-dependent transcription factors such as the nuclear factor of activated T-cells (NFAT) are attractive tools for synthetic biology applications. While ectopic expression of different mechanosensitive channels failed to enhance US stimulation of HEK293 cells, we overexpressed engineered NFAT variants to enhance calcium-influx induced activation of an exogenously introduced gene. The native NFAT DNA-binding domain was replaced with a designed TALE DNA-binding domain and the VP64 activation domain ensuring efficient calcium-dependent activation and elimination of off-target effects, demonstrating that the engineered NFAT-TALE chimera does not interfere with autologous NFAT-regulated genes. We demonstrate that insonation triggered expression of the pro-inflammatory cytokine human interleukin 10 *in vitro*. Further, US stimulation-triggered calcium entry activated a synthetic transcription factor in cells subcutaneously implanted in mouse. The engineered NFAT-based transcription factors characterised here are suitable for a range of therapeutic and diagnostic applications, as the calcium-dependent circuit can be coupled to diverse calcium signal transducing receptors and could be remotely activated with US.
Objectives:
The goal of this proposal was to develop the potential of ultrasound as a non-invasive and specific trigger for activation of target cells and induction of the transcriptional regulation of selected genes in mammalian cells by ultrasound stimulation. In short, we aimed to construct genetic tools to enhance the sensitivity of cells to ultrasound or other mechanical stimuli and couple mechanoreceptor activation to transcription of selected genes. Within the project, we specifically proposed:
(i) to enhance mechanosensing of cells to stimulation by ultrasound by introducing ectopic mechanosensitive ion channels;
(ii) to couple the ultrasound stimulation to signal transduction and transcriptional activation;
(iii) to demonstrate ultrasound stimulation of transcriptional regulation *in vivo*.
Results Obtained:
Regarding the first objective to enhance mechanosensing of cells to stimulation by ultrasound by introducing ectopic mechanosensitive ion channels several ion channels of mammalian and bacterial origin were expressed in HEK293 cells. They localised to the plasma membrane and were responsive to small molecule agonists or known mechanic triggers such as elevated or decreased temperature demonstrating their functionality. Ultrasound stimulation of transiently or stably transfected cell lines did not enhance stimulation over empty vector-transfected cells. This observed phenomenon was at least in part due to the expression of the Piezo1 channel. Nevertheless, we showed that ultrasound can induce a strong influx of Ca2+ in HEK293 cells and some other mammalian cell lines. The Ca2+ influx was later used for the specific triggering of gene regulation and expression.
Our second objective was to couple ultrasound stimulation to signal transduction and transcriptional activation. Calcium-dependent transcription factors are attractive tools for synthetic biology applications, as many different physical and chemical stimuli can induce the cellular uptake of calcium ions by activating a variety of membrane receptors, such as G-protein coupled receptors and calcium-selective ion channels. Most calcium-dependent synthetic gene circuits engineered to date in eukaryotic cells harness the nuclear factor of activated T-cells (NFAT) signalling pathway. We overexpressed NFAT in HEK293T cells to enhance the activation of an exogenously introduced gene. We found that NFAT overexpression results in its constitutive translocation and transcriptional activity in the absence of calcium influx. To enable potent NFAT activity while maintaining tight control, we prepared engineered NFAT variants with modified subcellular localisation properties. Furthermore, the native NFAT DNA-binding domain was replaced with a designed TALE DNA-binding domain and the VP64 activation domain. This replacement ensured efficient calcium-dependent activation of target transgene expression in several mammalian cell types. Replacement of the DNA-binding domain also eliminated off-target activation of NFAT promoter-driven gene expression, implying that the engineered NFAT-TALE chimera does not interfere with autologous NFAT-regulated genes. We showed that these engineered NFAT-TALE transcription factors can be stimulated in different cells, coupled to agonist or mechanic stimulus-induced TRP channel-specific Ca2+ influx or due to ultrasound stimulation. Reporter or therapeutic protein expressions were used as readouts.
As our last goal was to demonstrate ultrasound stimulation of transcriptional regulation *in vivo*. We showed that the above described synthetic device can upon implantation be triggered by ultrasound in live mice.

Results Unforeseen in the Original Project:
The high sensitivity of some cell lines to ultrasound in the absence of ectopic expression of mechanosensitive channels. Selectivity of ultrasound stimulation was achieved by utilising an engineered signalling pathway that does not interfere with endogenous pathways.

Publications:

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SRI LANKA

Title: Genetic dissection of polyethylene degradation ability of *Perenniporia* sp. isolated from decaying hard woods in Sri Lanka

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ICGEB Contract No.: CRP/19/14
ICGEB Reference No.: CRP/LKA18-03

Objectives:

Objectives of this project were to further sample for more fungal isolates from decaying hard woods and more species of the genus *Perenniporia*, to test the ability to grow on Polyethylene containing media, to identify the effective isolates up to the species level using molecular and morphological methods and to study the expression of selected ligninase genes when the mycelia are grown on polyethylene containing media.

Results Obtained:

All together 85 pure cultures of decaying wood associated fungi were obtained. Among them, three isolates belonged to the genus *Perenniporia*. Most of the isolates belonged to Ascomycetes. While the members of family Hypoxylaceae among Ascomycetes dominated as decaying wood associated fungi, family Phanerochaetaceae were dominated among Basidiomycetes. Among 50 randomly selected fungal isolates tested for their laccase production and lignin degradation abilities, 17 were laccase producers and 30 showed lignin degradation abilities as per qualitative assays. Repeated assays gave the same results. Even though laccase is a key enzyme for lignin degradation, it appears that the process is more complex than presumed. Most of the decaying wood associated fungi could utilize wood as the sole Carbon (C) source indicating that these fungi are indeed wood decay fungi. A modified method was developed for a large scale wood decay assays. Wood pieces inoculated with fungal isolates in liquid media showed that *Xylaria* feejeensis, and *Phlebiopsis* sp. were among the top three wood degraders. Percent weight loss ranged from 2.5% to 30%. All the isolates were assayed for their ligninase enzyme production. Two *Perenniporia tephropora* isolates were the highest laccase producers. Highest lignin peroxidase producers were *Arcopilus aureus*, Xylaria feejeensis and *Plebiopsis* sp. *Plebiopsis flavidoalba* and several other 20 isolates were subjected for polyethylene degradation assay. Parameters tested; weight loss, reduction in tensile properties, changes in contact angle, SEM (scanning electron microscopy) and FT-IR. Among tested isolates, *Plebiopsis* sp., Schizophyllum *commune*, *Fusarium* sp. *Phanerochaete* sp., *Xylaria* sp. were among the top five isolates. Low Density Polyethylene (LDPE) sheets incubated with *Plebiopsis* sp. showed significant changes FTIR spectrum with disappearance of some peaks and appearance of some peaks. In addition, peeling off thin layers of LDPE were also detected. Hence CO2 emission test was conducted for *Plebiopsis* incubated with LDPE and showed 3% degradation upon incubation. Molecular docking studies were conducted for lignin of *Schizophyllum commune* and lignin and polyethylene. Best model was found for laccase had lignin and polyethylene binding sites. C source, N source and the metal ion type and concentrations were optimised for the laccase production. It was found that starch and cellulose were best C sources, yeast extract was the best N source and CuSO4 is the best metal ion for laccase production. There was no significant positive correlation with polyethylene degradation and ligninase enzyme production. Full genome and transcriptome was obtained from *Plebiopsis* sp. in search of key genes of polymer degradation. Data analysis is ongoing. Results unforeseen in the original project: *Peraniporia* species were found to be the best laccase producer. Even though previously Peraniporia was the best polyethylene degrader in our preliminary analysis, *Plebiopsis* was found to be the best degrader when excessive sampling was done and extended the experiment for all the samples. Based on the previous records we presumed that lignocellulose enzyme has a role in polyethylene degradation. Our results did not find any significant positive correlation with degradation and enzyme production. However, molecular docking studies confirmed the presence of polyethylene binding sites in laccases. This indicates that somehow though not directly and significantly correlated, lignolytic enzyme have a role in degradation. NGS data of *Peraniporia* and *Plebiolpsis* is now available and molecular docking studies will be continuing. Transcriptome of the treated and non-treated samples will be a direct approach. However, for that we realised that no previous genomics data available for our best organism, *Plebiopsis flavidoalba*. Therefore, as the initial step Next Generation DNA data was obtained and now in the process of analysing. Isolation and purification of enzymes and characterisation will be the future direction. Thanks to the ICGEB, our next plan is the modification of media to achieve the best degradation while targeting a commercial product of fungal inoculum for plastic degradation.

Publication:

Perera, P., Deraniyagala, A., Mahawaththagea, M., Herath, H., Rajapakse, C., Wijesinghe, P., Attanayake, R. Decaying hardwood associated fungi showing signatures of polyethylene degradation. 2021. *BioResources* ***16*****(4)**, 7056-7070

TANZANIA

Title: Inter-epidemic transmission dynamics of Rift Valley, Dengue and Chikungunya viruses in Tanzania

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ICGEB Contract No.: CRP/19/011

ICGEB Reference No.: CRP/TZA18-04

Abstract: Little is known about the endemic burden of arthropod-borne disease-causing viruses (Dengue, Rift Valley, and Chikungunya) during inter-epidemic (IEPs) times. We wanted to know how common Dengue, Rift Valley, and Chikungunya were in humans, goats (RVFV), and mosquitoes (DENV, CHIKV, and RVFV) in Kilimanjaro's Lower Moshi area. Understanding the virus' maintenance mechanisms throughout inter-epidemic periods (IEPS) is vital, particularly understanding where the viruses hide during "quiet" times.

We performed repeated seasonal cross-sectional surveys in different seasons of the year, for 24 months, in a unique environment with strong human-mosquito-livestock interactions: the Kilimanjaro region's Lower Moshi area. The location has close human-livestock-mosquito interaction, making investigations on the transmission of RVFV, DENV, and CHIKV arboviruses possible. For comparison purposes, we also gathered equivalent data from two additional geographically distinct sites: Magugu (Manyara area) and Wami-Dakawa (Morogoro region). Seasonal mosquito, animal, and human viral infection rates, as well as arbovirus (RVFV, DENV, and CHIKV) seropositivity in humans and animals, were all investigated.

Objectives:

The proposed study therefore addressed the following broad goals:

(i) To determine seasonal infection rates of RVFV, DENV and CHIKV in Aedes (and Culex for RVFV) mosquitoes, ruminants (sheep and goats) and humans;

(ii) To determine seasonal sero-conversion rates to RVFV, DENV and CHIKV in ruminant animals (sheep and goats) and humans in study sites;

(iii) To establish significant geospatial clusters and modelling of RVFV, DENV and CHIKV distribution;

(iv) To build baseline human, arthropod and animal arbovirus surveillance capacity that will enhance future detection of emerging or re-emerging zoonotic vector-borne diseases.

Results Obtained:

Among the studied viruses, the Dengue virus is relatively uncommon, whereas Chikungunya and Rift Valley Fever viruses are far more frequent. This is supported by serological and molecular detection of the viruses in humans, animals (goats), and mosquitoes. Throughout the study period, goats were positive for RVFV antibody by 23.3%, as compared to humans (13.2%). By the use of qPCR, goats had the highest RVFV infection rate of 4.1%, followed by humans (2.6%), *Aedes aegypti* (2.3%), and Cx. pipiens complex (1.5%). In absence of recent data on RVFV infection in the region, our data points to the Lower Moshi region as a possible Rift Valley hotspot for RVF. Goats had the highest infection rate, indicating their potential role in maintaining RVFV during inter-epidemic periods. Across the seasons, 1.5% of *Aedes aegypti* pools (of 50 mosquitoes) were infected by CHIKV while 0.3% were infected by DENV. CHIKV was found in 1.9% of Cx.pipiens mosquitoes. DENV was not detected in any of the Cx.pipiens mosquitoes. Infection with CHIKV has been observed to be consistently higher in both humans and mosquitoes. This observation implies that both DENV and CHIKV are actively prevalent in the Lower Moshi area of Kilimanjaro region. Our data point to the Lower Moshi area as a potential hotspot for the survival of the two viruses and other vector-borne viruses. There was no substantial variance in these infection and exposure trends, which might be attributed to the research site's suitability for year-round mosquito breeding.

Our conclusions are the following: The detection of DENV, RVFV, and CHIKV in both humans and vector mosquitoes confirms that the viruses are actively circulating in the Lower Moshi area of the Kilimanjaro region in Tanzania during IEPs. We also report goats as key maintenance hosts for RVFV during IEPs. Our findings point out the Lower Moshi area as a potential focal point for the maintenance of the arthropod-borne viruses and possibly other vector-borne viruses. Since passive surveillance of arthropod-borne viruses such as DENV, CHIKV, and RVFV is challenging in the absence of clinical features among humans and livestock, active surveillance is recommended and, where resources may be limited, targeted surveillance in high-risk areas (hotspots) will help prevent future outbreaks. It is critical, therefore, to revisit the national contingency plans used in surveillance of emerging and re-emerging viruses and preparedness to respond to outbreaks, keeping in mind that active IEPs transmission of the viruses occurs in the absence of expected overt clinical manifestations that can be used as pointers to infections.

Results Unforeseen in the Original Project:

(i) Unexpectedly, none of the studied viruses (DENV, CHIKV, and RVFV) was detected in nearby sites from the study site, despite the presence of suitable ecology for mosquito breeding. This mosquito sterility warrants future investigations that will involve entomological studies.
(ii) Furthermore, it was unexpected to detect RVFV in humans, goats, and mosquitoes at the observed frequencies in the Lower Moshi area. Plans are underway to further characterise the viruses to understand their strain characteristics and phylogenetics.

Publications:

Budodo, R.M., Horumpende, P.G., Mkumbaye, S.I., Mmbaga, B.T., Mwakapuja, R.S., Chilongola, J.O. Serological evidence of exposure to Rift Valley, Dengue and Chikungunya Viruses among agropastoral communities in Manyara and Morogoro regions in Tanzania: A community survey. 2020. PLoS Negl. Trop. Dis**. 14(7)**, e0008061

Kumalija, M.S., Chilongola, J.O., Budodo, R.M., Horumpende, P.G., Mkumbaye, S.I., Vianney, J.M., Mwakapuja, R.S., Mmbaga, B.T. Detection of Rift Valley Fever virus inter-epidemic activity in Kilimanjaro Region, North Eastern Tanzania. 2021. Global Health Action **14(1)**, 1957554

TRINIDAD & TOBAGO

Title: Genetic contributors to Diabetes Mellitus in Indo-Trinidadians
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ICGEB Contract No.: CRP/16/019
ICGEB Reference No.: CRP/TTO16-04
Abstract: The aim of this project is to investigate the genetic causes of monogenic Diabetes Mellitus (DM) in the East Indian sub-population of Trinidad and to determine whether this disease has novel genetic contributors in this ethnic group. To date, there has been no genetic study into the causes of Diabetes in the Trinidadian population, yet the prevalence of this disease is one of the highest in the world (14%) and the mortality rate in Trinidad & Tobago is 14% compared to 11.3% globally. The East Indian sub-population accounts for 34% of the population and is one of the major ethnic groups within society with a disproportionately higher rate of DM. This increased prevalence of DM may allude to a potential founder effect as this population has tripled (~468,524 persons) since the first introduction of individuals to the island (~148,000 persons) from the Indian states of Bihar and Uttar Pradesh beginning in 1845. Prevalence of monogenic DM is unknown in Trinidad and may be misdiagnosed for the more common Type 1 and Type 2 DM. This is clinically important as it may affect treatment of patients and at-risk relatives. This population presents a unique and understudied population for the elucidation of novel variants that may contribute to the diabetic phenotype.
In order to identify novel or known variants that are associated with monogenic DM in Trinidad, 400 participants with DM from diabetes clinics across the island were interviewed and a venous blood or saliva sample was collected. Genomic DNA was extracted using commercially available kits and stored. Families of persons who have a strong family history and display a Mendelian inheritance pattern without the usual risk factors for DM were studied. Whole exome sequencing (WES) was done on two distantly related affected individuals per family using an Illumina platform (100X coverage). Reads were mapped using BWA and duplicates were flagged using Picard Mark Duplicates. Variants were called using GATK and annotated using SnpEff. Filtering and prioritisation were done within the Qiagen Ingenuity Variant Analysis software. Variants were then screened in additional family members by PCR and Sanger sequencing.
To date, 34 families have been identified for further study and 5 have undergone WES. Candidate variants in 14 genes have been identified. One of these genes has previously been implicated in MODY 4 and five others have previously been associated with other forms of DM. Thus, eight potentially novel genetic contributors to the diabetes phenotype have been identified in this population thus far. Validated variants will be screened in the entire cohort of samples. Based on the validated variants identified, a targeted NGS based diagnostic panel specific for this population would be designed. Investigation of additional families is ongoing.
Overall this study is expected to serve as demonstration of how genetic screening may play a key role in the health care system of Trinidad and Tobago for managing a major public health issue. Furthermore, it will introduce the implementation of cutting-edge genomic technologies for developing personalised medicine initiatives in the Caribbean. Our results suggest that there may be novel genetic contributors to this phenotype in this population.
Objectives:
In order to investigate the underlying genetics of monogenic forms of Diabetes in the East Indian subpopulation of Trinidad, we proposed a pilot study which was the first of its kind in Trinidad and Tobago to use whole exome sequencing to map the causative variants of monogenic diabetes in up to 20 pedigrees which display a Mendelian pattern of inheritance of Diabetes. The specific aims are as follows:
(i) To select 20 Indo-Trinidadian families displaying a Mendelian inheritance pattern for Diabetes Mellitus and biobank DNA samples from all family members;
(ii) To map causative genetic variants in each family using Whole Exome Sequencing (WES) and verify results using Sanger sequencing;
(iii) To determine a set of variants and genes including known and novel genes discovered to be associated with MODY to design a screening panel for future studies in this country.
Results Obtained:
Four hundred (400) whole blood/saliva samples from chronic disease clinics across Trinidad were collected and biobanked and all study participants were extensively interviewed. Genomic DNA was extracted from all samples and banked. Participants provided informed consent to a 10-year limit on storage of these samples. Thirty-four (34) family pedigrees displaying a Mendelian inheritance pattern for Diabetes Mellitus were identified for further study. Two samples each from five (5) families have undergone whole exome sequencing (WES) thus far. A bioinformatics pipeline using standard analysis algorithms for whole exome sequencing data was implemented in the Galaxy open-source bioinformatics environment to align sequencing data to the human genome and call variants.

Ingenuity Variant Analysis (Qiagen) was then used for variant filtering and prioritisation. To date, variants in 14 genes have been identified as potential genetic contributors to the phenotype. Interestingly, only one of these genes (PDX1) has previously been associated with MODY and five others have been previously associated with other forms of DM. Thus, we have identified eight novel candidate genes that may underlie the MODY phenotype in this population. Validation of these findings and analysis and screening of additional families are in progress.

Results Unforeseen in the Original Project:

Our initial hit rate of 34 pedigrees out of 400 displaying a Mendelian inheritance pattern for Diabetes Mellitus was surprising. We initially estimated that we would need to screen 2000 probands in order to identify 20 families based on conservative estimates of a prevalence of 1% monogenic diabetes in the United States of America from the American Diabetes Association. This result indicates that the prevalence of MODY in this population could potentially be much higher than that observed in the US population (1-5%) and even as high as 8.5% in the Indo-Trinidadian population.

URUGUAY

Title: Decoding the molecular interaction between the plant growth promoting bacteria *Streptomyces* sp. UYFA156 and it host *Festuca arundinacea*

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ICGEB Contract No.: CRP/17/013
ICGEB Reference No.: CRP/URY17-01

Abstract: *Streptomyces* sp. UYFA156 was isolated from the inside of surface sterilised seeds of *Festuca arundinacea* (tall fescue) cultivar (cv.) SFRO Don Tomás. The isolate presents specific growth promotion activity (PGP) on its natural host, but not on the tall fescue cv. Tacuabé.

This proposal aims to elucidate the underlying molecular mechanisms in the *Streptomyces*-tall fescue interaction. By confocal microscopy we described the colonisation and infection process by UYFA156 in both cvs, being a true endophyte of them. Using massive sequencing of the 16S rRNA gene we described the modulation of the endophytic microbiome associated to both cvs. upon inoculation with UYFA156. The growth promotion by UYFA156 is accompanied by an early effect in the diversity and composition of the endophytic microbiome in a host-specific manner. RNAseq technique was employed to study the effects of UYFA156 on a sympatric strain, *Microbacterium* sp. UYFA68. Several genes were identified as differentially expressed in UYFA156, many of which are likely involved in the modulation of the composition and activity of the endophytic microbiome associated with tall fescue plants. Differentially expressed genes were also identified in UYFA68 upon exposure to UYFA156. Interestingly, the IAA production of UYFA68 is modulated by UYFA156 and the role in the PGP of tall fescue plants is under study.

Objectives:

The general objective of this research was to characterise the molecular bases of the interaction between the strain *Streptomyces* sp. UYFA156 and tall fescue cv. SFRO Don Tomás plants, while the specific objectives were the following:

(i) To determine if the strain UYFA156 is a true endophyte of tall fescue cultivars grown in Uruguay;
(ii) To determine if the BNF is one of the PGP mechanisms involved in the PGP effect observed in tall fescue cv. SFRO Don Tomás;
(iii) To determine at least two genetic components probably involved in the colonisation and/or PGP of tall fescue plants by the strain UYFA156.

Results Obtained:

(i) Strain *Streptomyces* sp. UYFA156 is a true endophyte of the two tall fescue cultivars studied: SFRO Don Tomás (DT), which is its natural host; and Tacuabe (Ta), a commercial cultivar.
(ii) The differential effects in PGP observed between cvs. upon UYFA156 inoculation are not due to a differential colonisation process by the bacteria.
(iii) Strain UYFA156 is not diazotrophic, and traits not commonly associated to PGP were identified encoded in its genome. Genome analysis showed also that strain UYFA15 belonged to the genus *Streptomyces albidoflavus*.
(iv) Strain UYFA156 modulates the endophytic microbiota *in vitro* and *in vivo* when inoculated onto tall fescue plants.
(v) Growth promotion by UYFA156 is accompanied by an early effect in the diversity and composition of the endophytic microbiome in a host-specific manner.
(vi) Several genes were identified as differentially expressed in the strain UYFA156, many of which are likely involved in the modulation of the composition and activity of the endophytic microbiome associated with tall fescue plants. The role of these genes in the interaction with the microbiome as well as in the PGP of tall fescue by the strain UYFA156 are under study.
(vii) Differentially expressed genes were also identified in the strain UYFA68 upon exposure to UYFA156. This strain is also part of the endophytic microbiota associated with SFRO Don Tomás tall fescue and its IAA production is modulated by the PGP strain UYFA156. The role of those differentially expressed genes in (including IAA-production related genes), in the interaction with the microbiome as well as in the PGP of tall fescue plants are under study.

All together the results obtained in the project allowed to reach the general objective of the proposal.

Publications:

Vaz Jauri, P., Taulé, C., de los Santos, M.C., Fernandez, B.,Battistoni, F. Interactions between putatively endophytic bacteria and tall fescue (*Festuca arundinacea*): plant growth promotion and colonization in host and non-host cultivars. 2020. Plant and Soil **451,** 207–220

Vaz Jauri, P., Beracochea, M., Fernández, B., Battistoni, F. Whole-genome sequencing of *Streptomyces* sp. strain UYFA156, a cultivar-specific plant growth-promoting endophyte of *Festuca arundinacea*. 2019. Microbiol. Resour. Announc. **8(38)**, e00722-19

URUGUAY

Title: Novel mechanisms of inflammatory macrophage proliferation
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ICGEB Contract No.: CRP/19/019
ICGEB Reference No.: CRP/URY18-02
Abstract: We explored the hypothesis that CD40L is a co-factor for macrophage proliferation. This included the possibility that adding CD40L to cultures would allow macrophages to proliferate in response to IL-4, as they do *in vivo*. We determined that, contrary to B cells, (peritoneal of bone marrow-derived) macrophages do not respond to IL-4 plus soluble CD40L with proliferation *in vitro*. In fact, cell-bound CD40L is available in resident peritoneal cell cultures, in which it appears to foster macrophage proliferation driven by M-CSF and certain responses to IL-4; this is not imitated by soluble CD40L. *In vivo*, endogenous CD40L is not important for IL-4-stimulated proliferation of resident or recruited peritoneal macrophages or adipose tissue macrophages in lean animals, or for the endogenously stimulated proliferation of adipose tissue macrophages in obesity (15 weeks of high-fat diet). However, endogenous CD40L does contribute to *in vivo* IL-4-driven macrophage proliferation in the case of liver Kupffer cells. Kupffer cell proliferation induced by endogenous IL-4 in a helminth infection model also depends heavily on CD40L.
Objectives:
(i) To determine in which of selected MΦ subtypes CD40L enhances IL-4-driven proliferation;
(ii) To define any stimuli other than IL-4 with which CD40L collaborates for MΦ proliferation;
(iii) To identify the CD40L receptor(s) relaying proliferative signals;
(iv) To assess if the CD40L stimulus for MΦ proliferation can be provided by cell-bound CD40L;
(v) To obtain information on mechanisms of CD40L collaboration with known proliferative signals;
(vi) To determine if blocking CD40L decreases MΦ proliferation in relevant *in vivo* models of pathology.
Results Obtained:
The following results have been achieved:
(i) Contrary to B-cells, resident or recruited peritoneal cavity macrophages or bone marrow-derived macrophages do not proliferate in response to IL-4 plus sCD40L, or IL-4 plus CD40 agonistic antibody.
(ii) The proliferation of macrophages in response to M-CSF in cultures of resident peritoneal cavity cells does not require endogenous CD40L, and it is inhibited by soluble recombinant CD40L.
(iii) Endogenous CD40L appears to be mostly dispensable for the proliferation of resident or recruited (thioglycollate-elicited) macrophages *in vivo* in response to exogenous IL-4.
(iv) Endogenous CD40L is dispensable for the proliferation of visceral adipose tissue macrophages *in vivo* in lean animals injected with IL-4.
(v) Endogenous CD40L is not important for the proliferation of F4/80hi visceral adipose tissue macrophages after the establishment of obesity (15 weeks of high-fat diet).
(vi) Endogenous CD40L is very important for the *in vivo* proliferation of Kupffer cells in response to exogenous IL-4.
Results Unforeseen in the Original Project:
(i) Endogenous CD40L appears not to collaborate towards the expression of the M(IL-4) marker Relm-α by resident macrophages in cultures of resident peritoneal cavity cells or *in vivo*. Soluble CD40L inhibits Relm-α expression by peritoneal macrophages *in vitro*.
(ii) The reason for the lack of a proliferative response to IL-4 *in vitro* by resident peritoneal macrophages does not appear to be the supra-physiological O2 tension normally used in cell culture. An *in vitro* proliferative response to IL-4 also does not appear to be restored in the presence of peritoneal cavity fluid, serum or plasma.
(iii) Endogenous CD40L appears to restrict the proliferation of peritoneal macrophages and their expression of the M(IL-4) marker Relm-α in the context of Heligmosoides polygyrus infection (which induces an IL-4 response in the peritoneal cavity and other sites).
(iv) Endogenous CD40L appears to be necessary for the *in vivo* proliferation of Kupffer cells in the context of Heligmosoides polygyrus infection (which induces an IL-4 response in the liver and other sites).
Publications:
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VIET NAM

Title: Identification of target autoantigens for Narcolepsy type I

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ICGEB Contract No.: CRP/19/012

ICGEB Reference No.: CRP/VNM18-05_EC

Abstract: Sleep interacts with many physiological functions including neuroendocrine, metabolic, cardiovascular and neurobehavioral traits. Narcolepsy type I (NT1) is an enigmatic sleep disorder in which orexin-producing neurons within the lateral hypothalamus are selectively eliminated, leading to low levels of orexins in the brain and cerebrospinal fluid (CSF). Although many studies suggested the autoimmune basic of NT1, the antigens responsible for immune cell-mediated orexin neuron destruction have not been identified. To date, the only biomarker-based diagnostic test for NT1 is measuring orexin-A levels in CSF that is invasive, time-consuming, expensive and side effect associated method.

In this study, we used a random human peptide-displayed phage library to screen the peptides which bind to autoreactive antibodies specifically in the NT1 patient blood serum. It turns out that phage display approach failed to select the peptide specifically bind to IgG obtained from NT1 patients, thereby the potential autoantigen(s) triggering orexinergic neuron destruction could not be identified. However, from this study, we have successfully established the first human induced pluripotent stem cell (hiPSC) line in Viet Nam, evaluated its characterisation and differentiated into orexin neuron. These results will allow us to establish the sleep in a dish model of NT1 and transplant iPS-derived orexin neurons into our preclinical model of narcolepsy to explore whether this cell replacement therapy can recover narcoleptic symptoms. Altogether, this study sets up an initial steps and tools to not only improve our understanding of neuronal mechanisms that regulate sleep, but also serve as a strong basis for developing a novel cell therapy for treatments of human narcolepsy.

Objectives:

(i) To identify the potential autoantigen(s) and autoreactive T cells triggering orexin neuron destruction based on phage display and induced pluripotent stem cells technology

(i.a) To identify peptides specifically bind to IgG obtained from NT1 patients: We screened a phage displayed random peptide library against pooled serum IgGs obtained from 10 Narcolepsy type I (NT1) patients using substrative biopanning strategy. Next, we confirmed the reactivity of 101 randomly selected phage clones to pooled sera from NT1 patients compared to healthy donors using ELISA and selected the phage clones that specifically bound to the pooled sera from NT1 patients. We validated the binding capacity and specificity of the selected positive phage clones with individual serum from 20 patients and 20 matched healthy controls using ELISA.

(i.b) To examine the expression of potential autoantigens on hiPSC-derived orexin neurons: We differentiated hiPSCs into orexin neurons using SDIA+BMP4 medium supplemented with ManNAC and characterised the hiPSC-derived orexin neurons by evaluating the expression and secretion of orexin using RT-PCR and ELISA, respectively.

(i.c) To identify the autoantigen-specific pathogenic T cells in NT1: Because the phage display approached could not identify a peptide that can specifically bind to IgGs of NT1 patients, in the next study we will use transcriptomic and proteomic approaches to identify the mRNAs and proteins specifically expressed in hiPSC-derived orexin neurons but not in the hiPSC-derived MCH neurons.

(ii) To develop a novel serological biomarker for NT1 based on the peptides selected by phage display

(ii.a) To identify the potential serological biomarker(s) for NT1: We used the selected NT1-specific peptides identified from content 1 to coat a ELISA plate, assessed the ability of the peptide to discriminate patients from matched healthy controls.

(ii. b) To evaluate the diagnostic performance of the potential NT1 serological biomarker(s) in the validation cohort: We collected a validation cohort with selecting criteria and performed peptide ELISA to evaluate ability of the selected peptide(s) to efficiently discriminate NT1 patients from healthy controls and evaluate diagnostic ability of the ELISA test.

Results Obtained:

(i) Peptides selected by phage display

A total of 101 selected peptides were tested for immunoreactivity evaluation by phage-ELISA. Although most peptides were unable to discriminate NT1 patients from healthy controls, our results suggested that the antibodies binding to isolated phage clones might not specifically be associated with NT1 patient's serum but rather some common types of antibodies developed during patient's lifetime.

(ii) hiPSC generation and characterisation

We have established the original footprint-free Vietnamese human induced pluripotent stem cell line from cord-blood derived CD34+ hematopoietic stem cells under cGMP-compliant process. This cell line expressed the majority of the pluripotent markers and differentiated into derivatives of three

germ layers. The availability of Vietnamese iPSC line could contribute to improvement of inadequate genetic diversity in the currently available hiPSC lines.

(iii) hiPSC derived Orexin neurons

We differentiated hiPSC to orexin neurons using the BMP4+ ManNAC+ method described by Hayakawa et al. Next, we characterised the hiPSC-derived orexin neurons using an immunofluorescence assay for OREXIN-A and demonstrated the expression of orexin at mRNA and protein levels.

Results Unforeseen in the Original Project:

From this study, we have established for the first time in Vietnam the hiPSC platform with the ability to generate foot-print free hiPSCs from peripheral and umbilical cord blood under a feeder-free system. Furthermore, we have successfully derived the hiPSC into orexin neurons, allowing to establish the sleep in a dish model for NT1 for further transcriptomic and proteomic studies or even transplant hiPSC-derived orexin neurons into our preclinical model of narcolepsy to develop cell therapy for NT1. Altogether, the hiPSC lines established from this study sets up an initial steps and tools to not only improve our understanding of neuronal mechanisms that regulate sleep, but also serve as a Vietnamese healthy control model for physiological processes and drug screening, especially when there is an inadequate genetic diversity in the currently available hiPSC lines.

Publications:

Tran, T.T., Nguyen, T.H., Nguyen, T.T., Nguyen, X.H. Establishment of a Vietnamese ethnicity induced pluripotent stem cell line (VRISGi001-A) from umbilical cord blood hematopoietic stem cells under a feeder-free system. 2021. Stem Cell Res. **53**, 102345