EUROPEAN COOPERATION
IN SCIENCE AND TECHNOLOGY

BM1406: Ion Channels and Immune Response toward a global understanding of immune cell physiology and for new therapeutic approaches (IONCHAN-IMMUNRESPON)

WG3 MEETING
10-12th March 2016

Faculty of Pharmacy
University of Lisbon, Portugal

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Agenda of Lisbon Meeting
COST Action no. BM1406
Action Title: Ion Channels and Immune Response toward a global understanding of immune cell physiology and for new therapeutic approaches (IONCHAN-IMMUNRESPON)
Lisbon, Portugal from 9th March to 11th March 2016

Wednesday 9th March

14:00-16:00	Registration
16:00	Welcome address
16:30	Opening Lecture Dr. Pierre Martineau (Fr) Design and use of antibody libraries for the development of therapeutic antibodies
17:30	Pr. Thomas De Coursey (USA) title
18:20	Welcome drink

Thursday 10th March 2016

09:00	Lecture 1 Dr. Catelijne Stortelers, Ablynx (Ge) Therapeutic targeting of ion channels with Nanobodies
10:00	Dr. Ettrich R. Cation translocation in human ORAI channels: Modeling and simulations.
10:20	Coffee break / posters
10:40	Pinto Espinoza C. Nanobodies that antagonize the P2X7 ion channel ameliorate inflammatory diseases
11:00	Dr. Rupprecht A. A proton leak during proliferation?
11:20	Dr. Louvet C. Exploring the role of *Torid* cation fluxes in Th17 cells
11:40	Dr. Jawhari A. Native and functional isolation of ion channels for therapeutic purposes
12:00	Lunch

14:00	Lecture 2 Pr. Claude Malvy, Roussy Institute (Fr) Therapeutic siRNAs: principle of action and cell delivery by functionalized Nanoparticles.
15:00	Dr. Konu O. Identification and validation of the mRNA-miRNA coexpression network obtained from a CHRNA5 RNAi model in MCF7 cells
15:20	Dr. Majewski L. Transgenic mice overexpressing key store operated calcium entry proteins in neurons offer novel possibilities for studying calcium signalling in AD
15:40	Coffee break / Poster
16:00	Dr. Rottoli E. Signaling and metabolic reprogramming of effector/memory T cells by purinergic P2X7 receptor
16:30 18:30	MC Meeting H2020
20:00	Meeting Dinner

Friday 11th March 2016

	Lecture 3
09:00	Dr. Jean-François Liégois, Liège (Be) Chemical and biological tools to explore SK channel functions and therapeutic potentials
10:00	Dr. Quieroz M.J. Synthesis of heterocyclic compounds that may interact with ion channels producing an immune response
10:20	Dr. Martin-Sanchez F Novel blockers of P2X7 receptor-induced membrane permeabilization inhibit unconventional IL-1² release
10:40	Coffee break / Poster
11:00	Homerin G. "Synthesis of new ligands of the P2X7 receptor as potential treatments in IBD and cancers"
11:20	Dr. Rubartelli A. Proton-pump inhibitors inhibit pro-inflammatory cytokine production by monocytes/macrophages and protect mice from acute sepsis
11:40	Dr. Soveral G. Aquaporin therapeutic modulation: emerging targets for drug discovery
12:00	Dr. Katjar E. Resting potential of cancer stem cells
12:20	Opening Discussion

Design and use of antibody libraries for the development of therapeutic antibodies

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Recombinant antibody molecules have revolutionized therapy in several fields, particularly in cancer, inflammation and autoimmune diseases. The first generations of antibodies have been derived from mice molecules that were chimerized or humanized to avoid an anti-antibody response when injected in patients. To increase safety, several strategies have been now developed to directly obtain fully human antibodies, as, for example, transgenic mice with a humanized immunoglobulin locus, or in vitro selection methods based on libraries of human molecules. The latter strategy has many advantages when a precise specificity or antibodies against antigens very homologous between mouse and human are desirable. Phage display selection is one of the first developed methods and has proved to be very efficient and robust. The approach relies on the availability of an antibody library whose quality is paramount for an efficient selection. Such libraries have been obtained from natural repertoires of immunized or naive human donors, or designed and constructed in vitro to suit the objectives of the project [1,2]. Coupling synthetic antibody repertoires and phage display is currently one of the best approaches to isolate antibodies for therapeutic applications. Selection is not only possible against proteins or peptides but more importantly against whole live cells or even tissues. This allows the easy selection against proteins impossible or difficult to purify as GPCRs or ion channels that currently constitute the main targets of chemical drugs but are still poorly targeted by antibody-based therapies.

1. Philibert, P., Stoessel, A., Wang, W., Sibler, A.-P., Bec, N., Larroque, C., Saven, J.G., Courtête, J., Weiss, E. & Martineau, P. (2007). A focused antibody library for selecting scFvs expressed at high levels in the cytoplasm. *BMC Biotechnol.* **7**, 81
2. Robin, G., Sato, Y., Desplancq, D., Rochel, N., Weiss, E. & Martineau, P. (2014). Restricted Diversity of Antigen Binding Residues of Antibodies Revealed by Computational Alanine Scanning of 227 Antibody-Antigen Complexes. *J. Mol. Biol.* **426**, 3729–3743

Nanobodies® tackling the Kv1.3 ion channel

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Ion channels represent an attractive but challenging class of drug targets. Despite several approved drugs today, poor selectivity with the potential for significant toxicity or suboptimal efficacy is still a concern. Due to their extraordinary specificity, biologics offer an attractive strategy for overcoming these problems with the prospect to discriminate even closely related ion channel family members.
Nanobodies® are a novel class of antibody-derived therapeutic proteins based on single-domain antibody fragments. Ablynx has generated functional Nanobodies across a wide range of target classes, including ion channels, with a propensity to target cryptic epitopes, clefts and grooves. The excellent formatting flexibility allows full control over valency and multi-specificity, and makes the Nanobody platform ideally suited as a discovery platform to tackle difficult targets like ion channels.
Ablynx has generated Nanobodies against Kv1.3, an ion channel that is critical for T cell activation with a classical 6 transmembrane topology. The current leads show picomolar binding affinities and potencies in a bioassay on primary T cells, with broad species crossreactivity. Using electrophysiology, Nanobodies with various functional profiles were characterized and excellent current blocking capacity was demonstrated. Additionally, the selectivity of the leads was evaluated on a set of highly related ion channels. Electrophysiology-based counter screens demonstrated more than 10 000 fold selectivity over the closest related Kv1 family members.
Additionally, we exploited the power of the Nanobody platform by generating several multivalent combinations, with extended half-life. We demonstrated that potency can be increased (~1000 fold) and that different functional profiles can be obtained. *In vivo* efficacy was demonstrated in a delayed type hypersensitivity (dermatitis) model. Therefore Kv1.3 Nanobodies are therapeutical candidates with wide applicability to autoimmune and inflammatory diseases.

Cation translocation in human ORAI channels: Modeling and simulations.

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Rainer Schindl,[2] Rudiger H. Ettrich[1,3]

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Ca^{2+}-release-activated Ca^{2+} channels, encoded by Orai channels, form an ubiquitous cellular Ca^{2+} entry pathway, and control diverse signalling processes including gene expression, cell proliferation and T-cell activation. The human genome contains three Orai isoforms; however it remains unknown if their sequence variations are required for specific Ca^{2+} signals. Orai1 senses the amount of cholesterol in the plasma membrane and apparently the interaction of Orai1 with cholesterol inhibits its activity, thereby limiting store-operated calcium entry [1]. High affinity Ca^{2+} binding to the pore entrance of Orai channels creates a local extracellular calcium accumulating region CAR and provides fundamental insight into the unique mechanism of Ca^{2+} permeation of Orai channels [2]. The combination of computational modeling of Orai channels and molecular dynamics simulations provided by the team in Nove Hrady, and functional patch clamp, site-directed mutagenesis and experimental biophysical experiments performed by the Linz Team allows to propose that the Orai1 channel architecture with a close proximity of CAR to the selectivity filter, which enables Ca^{2+}-selective ion permeation, enhances the local extracellular Ca^{2+} concentration to maintain Ca^{2+}-dependent gene regulation even in environments with relatively low Ca^{2+}concentrations [2].

1. Isabella Derler, Isaac Jardin, Peter B Stathopulos, Martin Muik, Marc Fahrner, Vaselina Zayats, Saurabh Pandey, Michael Poteser, Barbara Lackner, Marketa Absolonova, Klaus Groschner, Rüdiger Ettrich, Mitsu Ikura, Christoph Romanin (2016) **Cholesterol binding modulates Orai1 channel function** *Science Signaling* 9 (412): ra10. Jan 26.

2. Irene Frischauf, Vasilina Zayats, Michael Deix, Anna Hochreiter, Isaac Jardin Polo, Martin Muik, Barbara Lackner, Barbora Svobodova, Teresa Pammer, Monika Litvinukova, Amrutha Arumbakam Sridhar, Isabella Derler, Ivan Bogeski, Christoph Romanin, Rüdiger Ettrich, Rainer Schindl (2015) **A calcium accumulating region, CAR, in the Orai1 channel enhances Ca2+- permeation and SOCE-induced gene transcription** *Science Signaling* 8 (408): ra131. Dec 22.

Nanobodies that antagonize the P2X7 ion channel ameliorate inflammatory diseases

Welbeck Danquah[1*], Catherine Meyer-Schwesinger[2*], Björn Rissiek[1,3*], Carolina Pinto[1], Arnau Serracant-Prat[1], Stephan Menzel[1], Eva Tolosa[1], Tim Magnus[3], Friedrich Haag[1], Toon Laeremans[4], Catelijne Stortelers[4], Friedrich Koch-Nolte[1]
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P2X7, a nucleotide-gated plasma membrane ion channel, responds to ATP released from cells as a danger signal during inflammation. Gating of P2X7 results in the production of the pro-inflammatory cytokine IL-1β by macrophages, whereas on T cells it induces exposure of phosphatidylserine and ectodomain shedding of L-selectin CD62L and CD27. Furthermore, it triggers the opening of a non-selective pore (pannexin 1) which can lead to cell death (1). Sustained activation of P2X7 is associated with chronic inflammatory processes and autoimmune diseases. Therefore, P2X7 represents an excellent target for the development of therapeutic antibodies in inflammatory disorders (2). Here, we report the generation of Nanobodies (the smallest antibody fragment) (3) as specific antagonists of P2X7. We isolated a Nanobody that blocks effectively P2X7 activation, inflammasome activation, release of IL-1β and death of Tregs. We show that a bivalent format of this Nanobody is much more potent than previously described P2X7-antagonistic antibodies and small molecule inhibitors. *In vivo*, a half-life extended version of this Nanobody ameliorates experimental glomerulonephritis and allergic dermatitis. Our results confirm P2X7 as an excellent target for Nanobody-based therapeutics.

1. Idzko M, Ferrari D, Eltzschig HK (2014). Nucleotide Signalling during inflammation. Nature. 509:310-317.
2. Bartlett, R., Stokes, L., and Sluyter, R. (2014). The P2X7 Receptor Channel: Recent Developments and the Use of P2X7 Antagonists in Models of Disease. Pharmacol. Rev. 66:638-675.
3. Wesolowski, J., Alzogaray, V., Reyelt, J., Unger, M., Juarez, K., Urrutia, M., Cauerhff, A., Danquah, W., Rissiek, B., Scheuplein, F., et al. (2009). Single domain antibodies: promising experimental and therapeutic tools in infection and immunity. Med. Microbiol. Immunol. 198:157-174.

This abstract fits best into (please underline): WG1 (cells), WG2 (diseases), WG3 (drugs)

A proton leak during proliferation?

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Activated lymphocytes reprogram their metabolism to aerobic glycolysis to support their clonal expansion. We found that the expression of mitochondrial uncoupling protein 2, a member of the solute carrier family 25, correlates with the metabolism of high proliferating cells (1,2). Using an evaluated antibody we showed that UCP2 protein is present in all tissues and cells associated with the immune system (1). The function of UCP2 in T-cells is not clear. Stimulation and re-stimulation of T-cells lead to an increase in UCP2 expression which correlates with cell proliferative and metabolic activity (Fig. 1). By investigating a recombinant UCP2 reconstituted in lipid membranes, we have demonstrated that UCP2 mediates a proton leak akin to UCP1 (3), the mediator of the non-shivering thermogenesis. There is a 200 times lower occurrence of UCP2 in comparison to UCP1 which implies that it is considerably involved in mild uncoupling and regulation of reactive oxygen species rather than in thermogenesis. This putative function has a protective role in T-cells.

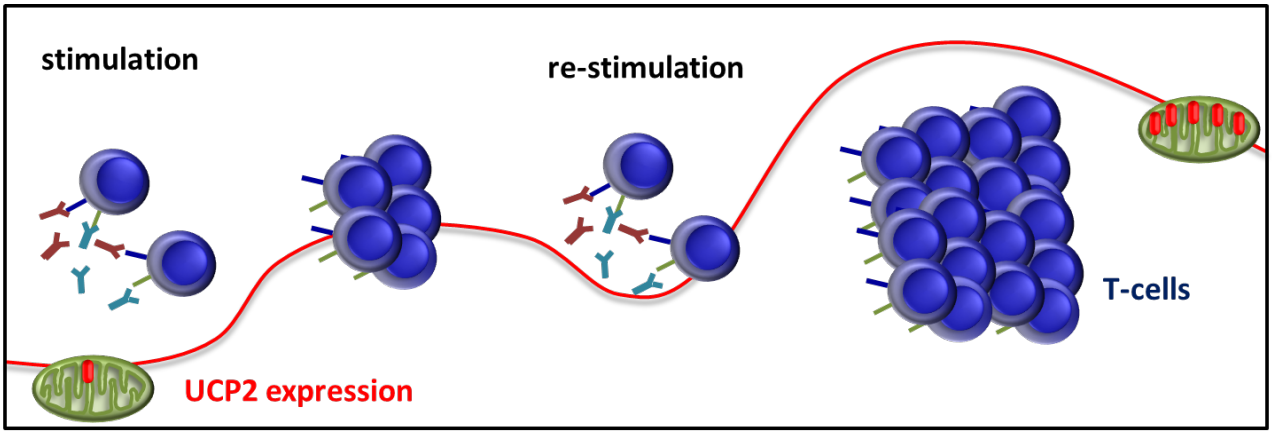

Fig. 1 Schematic diagram of the UCP2 up-regulation during T-cell stimulation

1. Rupprecht,A. et al. PLoS One. 2012;7(8).
2. Rupprecht, A. et al. PLoS One. 2014 Feb 11;9(2).
3. Rupprecht,A. et al. Biophys J. 2010 Apr 21;98(8).

This abstract fits best into (please underline): WG1 (cells), WG2 (diseases), WG3 (drugs)

Exploring the role of *Torid* cation fluxes in Th17 cells

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Tmem176b, initially named *Torid* (*tolerance-related and induced*) (Louvet et al. 2005), encodes a four-span transmembrane protein that we recently showed to be involved in antigen crosspresentation in Dendritic Cells (DCs) through acting as a non-selective cation channel that finely regulates the phagosomal pH (Segovia et al. 2014). However, growing evidence suggest that its function goes beyond such a restricted role in DCs. Importantly, *Tmem176b* has a co-regulated homologue, namely *Tmem176a*, located within the same genomic locus and likely resulting from a recent duplication. Surprisingly, we found that both genes are highly expressed in Th17 cells (Figure 1) (submitted manuscript). TMEM176A and B exhibit a similar cation channel activity and mainly colocalise in close proximity to the *trans*-Golgi network. Interestingly, *Tmem176b* single-deficiency in the mouse partially but significantly reduces imiquimod-induced psoriasis-like skin inflammation. These findings shed light on a potentially novel specific process linked to post-Golgi trafficking for modulating the function of Th17 (and more generally RORγt$^+$ cells) and indicate that both homologues should be simultaneously targeted to clearly elucidate the role of this intracellular ion flow. In this regard, we have recently successfully generated a double KO mouse using the CRISPR technology.

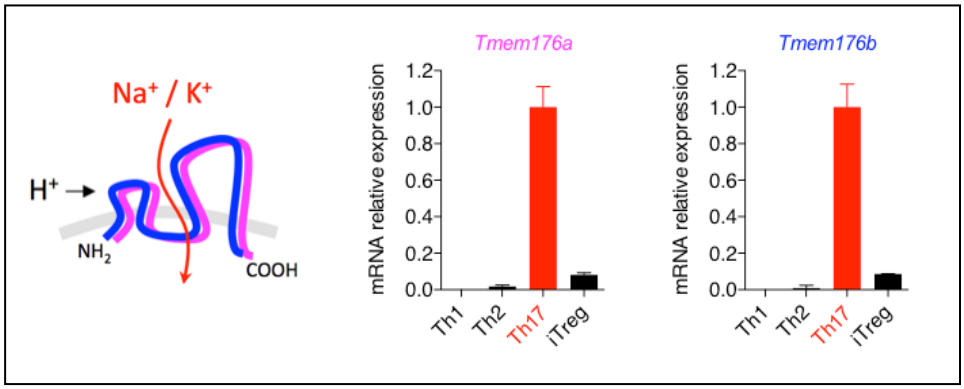

Figure 1. Schematic representation of the four-span transmembrane protein TMEM176B acting as a non-selective cation channel activated by acidity and strongly expressed in Th17 cells.

Louvet, C., Chiffoleau, E., Heslan, M., Tesson, L., Heslan, J.M., Brion, R., Beriou, G., Guillonneau, C., Khalife, J., Anegon, I., et al. (2005). Identification of a new member of the CD20/FcepsilonRIbeta family overexpressed in tolerated allografts. Am J Transplant 5, 2143-2153.

Segovia, M., Louvet, C., Charnet, P., Savina, A., Tilly, G., Gautreau, L., Carretero-Iglesia, L., Beriou, G., Cebrian, I., Cens, T., et al. (2014). Autologous dendritic cells prolong allograft survival through Tmem176b-dependent antigen cross-presentation. Am J Transplant 14, 1021-1031.

Title: Native and functional isolation of ion channels for therapeutic purposes

Presenter: Anass JAWHARI, Chief Scientific Officer, CALIXAR.

Abstract:
Membrane proteins represents up to 70% of therapeutic targets that are involved in different key cellular processes and signaling events. Surprisingly, less than 1% of protein structures in the Protein Data Bank correspond to membrane proteins. Those targets are very often instable which represents a bottleneck for their production in homogenous and stable state suitable for structural studies, antibody development and HTS ligand screening. CALIXAR has developed a surfactant/ detergent based approach consisting on native isolation of therapeutic targets from the expression, solubilization/stabilisation, and purification to functional as well as structural characterization. Instead of modifying the protein (by truncations and mutations) to adapt to the environment, we modify the chemical environment to adapt to the protein, using innovative proprietary detergents/ surfactants compounds and combination of compounds to help solubilize and stabilize in the same time. The starting material can be either endogenous (Organs, Primary cells, bacteria, and viruses), or recombinant (*E.coli*, Yeast, insect cells, CHO and HEK cells).

Here we will describe examples of highly druggable targets including key ion channels that were produced in their most native state without any single mutation, truncation or fusion and that could exhibit striking thermo-stability increase and homogeneity improvement, while keeping their functional features.
In addition to that, CALIXAR approach was able to identify protein partners at the membrane since protein/ protein interactions are maintained while the target protein is extracted from the membrane. This helps better understand the mechanism of action. A specific example in the context of pancreatic cancer biology will be described (Rosati A *et al.,* Nature Communication, 2015).

SiRNA: Principle of action and improvement of cell delivery by using functionnalized nanoparticles.

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siRNA are double stranded 21 bases RNA oligonucleotides able to inhibit a given gene expression thanks to their interaction with the gene mRNA in the intracellular RISC complex. The sequence of one siRNA strand (the other one is Watson Crick complementary) is contained in the target mRNA. They trigger a specific mRNA degradation (behaving for this like antisense oligonucleotides) , therefore inhibiting the synthesis of the corresponding protein. This process has been shown to work *in vivo* in humans and several clinical trials are taking place with either siRNAs or antisense ologinucleotides (one antisense oligonucleotide, mipomersen, has been approved by FDA for treatment of hypercholesterolemia by subcutaneous delivery) . However siRNA for therapeutics present a double problem : 1) As polyanions their cell uptake is very poor.2) As nucleic acids they are quickly degraded in plasma. One possibility to overcome these problems is to deliver siRNA *in vivo* with nanoparticles. These nanoparticles will protect the siRNAs in plasma and act as a Trojan horse for the targeted cells. In order to reach *in vivo* a therapeutic concentration of siRNA in cells it is therefore necessary to optimize the intracellular delivery by nanoparticles. I shall describe the interaction of nanoparticles with different types of cells and then how to functionnalize these nanoparticles to improve cell delivery. This can be obtained by modifications of nanoparticles with 1) cell penetrating peptides to increase cell delivery, 2) With ligands of cell receptors to increase the specificity of nanoparticles: natural ligands, peptides, aptamers, antibodies. We can then speak of immunonanoparticles. Examples of applications with oligonucleotides and siRNAs will be given.

Hillaireau H., Couvreur P. Nanocarriers entry into the cell : relevance to drug delivery
Cell Mol. Life Sci. 66(17) 2873-96 (2009)

Steichen S., Caldorera-Moore M , Peppas N. A review of current nanoparticle and targeting moieties for the delivery of cancer therapeutics. European Journal of Pharmaceutical Sciences 48, 3, 416-427 (2013)

siRNA associated with immunonanoparticles directed against cd99 antigen improves gene expression inhibition in vivo in Ewing's sarcoma A. L. Ramon, J. R. Bertrand, H. de Martimprey, G. Bernard, G. Ponchel, C. Malvy and C. Vauthier
J. Mol. Recognit. 26: 318–329 (2013)

This abstract fits best into (please underline): WG1 (cells), WG2 (diseases), WG3 (drugs)

Identification and validation of the mRNA-miRNA coexpression network obtained from a CHRNA5 RNAi model in MCF7 cells

Ozlen Konu, Mehtap Yilmaz, Ermira Jahja, Basak Ozgursoy, Sahika Cingir, Huma Shehwana
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Research in our lab focuses on development of bioinformatics applications for comparative mRNA and miRNA transcriptome data in different tissues and in cancer (1-2). Cholinergic signaling is driven by acetylcholine and agonists like nicotine through interaction with nicotinic acetylcholine receptors, which are ligand-gated ion channels composed of homo- or heteromeric subunit combinations. Among the different subunits, Cholinergic Receptor, Nicotinic, Alpha 5 (CHRNA5), is differentially expressed in cancer cells and thus has become an important biomarker in lung cancer. However its role in breast cancer is not well understood. In the present study, using a microarray based transcriptomics analysis of mRNA-miRNA networks we showed that knock-down of CHRNA5 had significant anti-proliferative and immunomodulatory effects in the MCF7 breast cancer cell line. In addition, we validated significant changes in expression for selected mRNA-miRNA pairs modulated by CHRNA5 knock-down using qRT-PCR. Next we demonstrated that application of miRNA mimics in the presence or absence of CHRNA5 siRNA enabled testing of the potential synergism and/or antagonism for a given mimic-siRNA combination. As a result, we present for the first time a comprehensive analysis of mRNA-miRNA network modules upon CHRNA5 knock-down in MCF7 cells for determination of the functional contribution by CHRNA5-driven signaling in breast cancer. This study has been funded by grants (111T316, 114S367) from The Scientific and Technological Research Council of Turkey (TUBITAK).

1) Gur-Dedeoglu B*, Konu O*, Kir S, Ozturk AR, Bozkurt B, Ergul G, Yulug IG. A resampling-based meta-analysis for detection of differential gene expression in breast cancer. BMC Cancer. 8:396 (2008). *Equal contribution.
2) Kaya KD, Karakülah G, Yakicier CM, Acar AC, Konu O. mESAdb: microRNA expression and sequence analysis database. Nucleic Acids Res. 39(Database issue):D170-80 (2011).

This abstract fits best into (please underline): WG1 (cells), WG2 (diseases), WG3 (drugs)

Transgenic mice overexpressing key store operated calcium entry proteins in neurons offer novel possibilities for studying calcium signaling in AD

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Alzheimer disease (AD) is the most common form of adult dementia. The etiology of AD is unknown, however, age is its principal risk factor. One of the molecular hypotheses of ageing predisposing to AD is based on dysregulation of calcium homeostasis in aged brains. In cells from patients affected by sporadic Alzheimer's disease (SAD), the most common form of AD, disturbances in Ca^{2+} signaling are found before any obvious extracellular Aβ pathology. Moreover, Ca^{2+} dysfunction augments Aβ formation and Tau hyperphosphorylation. Our group has shown that the cytoplasmic resting Ca^{2+} level in cultured neurons can be modulated by overexpression of STIM proteins, ER Ca^{2+} sensors involved in the Store Operated Calcium Entry (SOCE) [1, 2]. Since SAD is believed to be a systemic disorder, changes might also occur in peripheral cells, such as fibroblasts or blood cells. We detected an enhanced magnitude of Ca^{2+} influx during SOCE in human lymphocytes from SAD patients [3], and decreased level of STIM2 protein in human lymphocytes from familial Alzheimer's disease (FAD) patients in parallel to an attenuation of SOCE [4]. We are now measuring Ca^{2+} signaling in cultured neurons and fibroblasts derived from transgenic mouse lines overexpressing STIM1, STIM2 and Orai1 proteins.

[1] Gruszczynska-Biegala J, Pomorski P, Wisniewska MB, Kuznicki J. Differential roles for STIM1 and STIM2 in store-operated calcium entry in rat neurons. PLoS One; 6(4):e19285 (2011)

[2] Majewski L, Kuznicki J. SOCE in neurons: Signaling or just refilling? Biochim Biophys Acta Biochim Biophys Acta. 1853(9):1940-52 (2015)

[3] Jaworska A, Dzbek J, Styczynska M, Kuznicki J. Analysis of calcium homeostasis in fresh lymphocytes from patients with sporadic Alzheimer's disease or mild cognitive impairment. Biochim Biophys Acta 1833:1692-9 (2013)

[4] Bojarski L, Pomorski P, Szybinska A, Drab M, Skibinska-Kijek A, Gruszczynska-Biegala J, Kuznicki J., Presenilin-dependent expression of STIM proteins and dysregulation of capacitative Ca2+ entry in familial Alzheimer's disease. Biochim Biophys Acta 1793:1050-7 (2009)

Signaling and metabolic reprogramming of effector/memory T cells by purinergic P2X7 receptor

Elsa Rottoli, Fabio Grassi

Adenosine triphosphate is an ubiquitous extracellular messenger, which activates purinergic P2 receptors in plasma membrane of eukaryotic cells and regulates many cellular functions ranging from survival and proliferation to apoptosis. Deletion of *p2rx7* encoding for ATP-gated ionotropic P2X7 receptor confers increased survival to T follicular helper (Tfh) cells in Peyer's patches (1). We show that analogously to Tfh cells, T effector/memory (TEM) display the same pattern of expression of P2X7 during activation and proliferation. Transmission electron microscopy revealed that *p2rx7*$^{-/-}$ TEM cells are characterized by an alteration of mitochondrial cristae and abnormal mitochondrial mass. We investigated whether this altered mitochondrial morphology correlated with altered functional features in TEM cells. The deletion of p2rx7 in TEM cells results in increased survival and proliferation both in vitro and in an in vivo model of homeostatic expansion of T cells in lymphopenic host. We could also show that the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of *p2rx7*$^{-/-}$ TEM cells are increased, indicating that both oxidative phosphorylation and glycolysis are more active. Altogether these results point to a role of P2X7 in regulating T cell responsiveness.

1) Proietti M., Cornacchione V., Rezzonico Jost T., Romagnani A., Faliti C. E., Perruzza L., Rigoni R., Radaelli E., Caprioli F., Preziuso S., Brannetti B., Thelen M., McCoy K. D., Slack E., Traggiai E., Grassi F. ATP-gated ionotropic P2X7 receptor controls follicular T helper cell numbers in Peyer's patches to promote host-microbiota mutualism. Immunity 20;41(5):789-801 (2014)

This abstract fits best into : WG1 (cells), WG2 (diseases), WG3 (drugs)

Chemical and biological tools to explore SK channel functions and therapeutic potentials

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SK channel subtypes are an attractive target in various diseases such as cognitive dysfunction, dopamine related disorders, cardiovascular disorders, cancer or parasitosis. TEA is known to interact in the pore by physically blocking ion flow. The high sensitivity of some channels to TEA is related to the presence of an aromatic amino-acid residue in the pore region. Because the pore of SK channels contains a valine residue, its sensitivity to TEA is low. VA and VF mutations have been carried out (1). Apamin is the prototypical SK channel blocker with a high affinity, a high blocking property but a low SK2/SK3 selectivity. A multidisciplinary approach combining single-channel measurements and mutagenesis showed that the interaction between apamin and SK channels is located in the outer pore region leading to a current block by an allosteric-like mechanism. Most of the modulators have been found by serendipity or following a large screening of molecules. A rational target-based ligand design is a difficult task due to the lack of structural information about the target. Currently, only the S5-S6 region has been built by using in silico techniques. Some subtile differences between SK2 and SK3 subtypes have been detected. Indeed, in the outer pore region, an asparagine or a histidine which is present in a similar position have been targeted to develop molecules to target this discriminant residue (2).

1) Dilly S, Philippart F, Lamy C, Poncin S, Snyders D, Seutin V, Liégeois J-F. The interactions of apamin and tetraethylammonium are differentially affected by single mutations in the pore mouth of small conductance calcium-activated potassium (SK) channels. Biochem Pharmacol 85:560-569 (2013).

2) Badarau E, Dilly S, Wouters J, Seutin V, Liégeois J-F. Chemical modifications of the *N*-methyl laudanosine scaffold point to new directions for SK channels exploration. Bioorg Med Chem Lett 24: 5616-5620 (2014).

This abstract fits best into (please underline): WG1 (cells), WG2 (diseases), WG3 (drugs)

Synthesis of heterocyclic compounds that may interact with ion channels producing an immune response

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From some years now my research group has been involved in the synthesis of differently functionalized thieno[3,2-*b*]pyridine derivatives. Some of the synthesized compounds (Fig. 1) were shown to be potential antitumorals (1) and/or antiangiogenics inhibiting in the latter case the posphorylation of the tyrosine kinase domain of the membrane receptor *Vascular Endothelial growth Factor Receptor 2* (VEGFR2) (2). The biological studies were performed in collaboration with IPATIMUP and Faculty of Medicine of the University of Porto, Portugal. Now we are interested in synthesizing new heterocyclic compounds that may interact with ion channels producing an immune response and in collaborating with colleagues that be able to do either the design of those compounds or the screening assays, in order to find potential drug candidates for the treatment of important diseases.

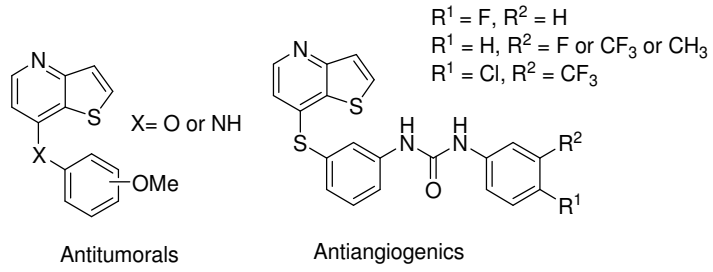

Fig. 1 Some heterocycle structures synthesized in our research group.

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This abstract fits best into (please underline): WG1 (cells), WG2 (diseases), WG3 (drugs)

Novel blockers of P2X7 receptor-induced membrane permeabilization inhibit unconventional IL-1β release

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P2X7 receptors (P2X7R) are cationic channels gated by extracellular ATP that are mainly expressed by immune cells. In myeloid cells, P2X7R induces opening of hemichannels leading to membrane permeabilization, reactive oxygen species (ROS) production, NLRP3-inflammasome activation and subsequent release of the pro-inflammatory cytokine IL-1β that follows an unconventional protein secretion mechanism. P2X7R has been implicated in several inflammatory disorders and the development of specific P2X7R antagonists by pharma industry has result in clinical trials for novel anti-inflammatory therapies. Punicalagin is a well-known antioxidant polyphenol compound isolated from pomegranate (Figure 1), we aimed to study punicalagin as a possible blocker of ROS production trigger by P2X7R and its possible effect on IL-1β processing/release. We found that punicalagin prevented the production of ROS and secretion of mature IL-1β induced P2X7R, but interestingly it did not affect IL-1β processing, NLRP3 activation, ASC oligomerization, caspase-1 activation or the release of IL-6 or TNF-α. Interestingly, punicalagin also blocked P2X7R induced plasma membrane permeabilization but not P2X7R cationic channel opening. These data identify punicalagin as a potential anti-inflammatory molecule able to specifically inhibit IL-1β secretion induced by P2X7R without affecting ion channel *per se* or NLRP3 inflammasome formation, but affecting ROS production and plasma membrane permeabilization.

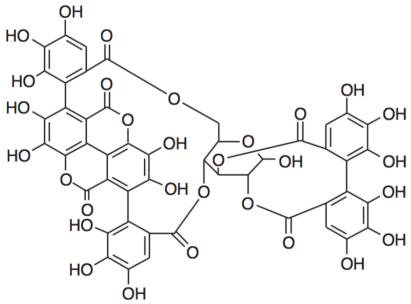

Figure 1. Chemical structure of punicalagin

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This abstract fits best into (please underline): WG1 (cells), WG2 (diseases), WG3 (drugs)

Proton-pump inhibitors inhibit pro-inflammatory cytokine production by monocytes/macrophages and protect mice from acute sepsis

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Sepsis is increasing representing a tremendous burden for health-care systems. Death in acute sepsis is attributed to hyperinflammatory responses (huge release of ATP and pro-inflammatory cytokines, 1,2). As acidosis is a common trait of septic patients, we explored proton pump inhibitors (PPI, such as esomeprazole) that block gastric acid secretion, as therapeutic agents in sepsis. We found that *in vitro* PPI inhibited TNF-α and IL-1β secretion by TLR-activated monocytes, at low and neutral pH. *In vivo*, esomeprazole i.p. protected mice from endotoxic shock (60% survival vs 5% of untreated mice) and decreased TNF-α and IL-1β systemic production. Esomeprazole was efficacious even if administered when the serum peak of TNF-α was already reached, indicating that cytokine suppression is not the only therapeutic mechanism. Esomeprazole-treated survived mice developed a long-term cross-tolerance, becoming resistant to LPS and other TLR agonists.
Therapeutic effectiveness, lack of toxicity, low cost, availability in most countries indicate PPI as promising new drugs against sepsis and other severe inflammatory conditions. It remains to be identified the molecular target(s) of PPI as anti-inflammatory drugs. Inflammatory cells lack the gastric H^+/K^+ ATPase proton pumps but express v-ATPases on the plasma membrane. However, whether and how PPI inhibit v-ATPases is unclear.

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This abstract fits best into (please underline): WG1 (cells), WG2 (diseases), WG3 (drugs)

Aquaporin therapeutic modulation: emerging targets for drug discovery

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Aquaporins (AQPs) are membrane channels involved in the bidirectional transfer of water and small solutes across cell membranes that have been progressively identified as key players in several physiological mechanisms. AQPs dysfunction or aberrant expression has been implicated in disease, suggesting a great translational potential in aquaporin-based diagnostics and therapeutics [1].

AQPs are highly expressed in different tumor types, where they are involved in tumor invasion, metastasis and growth [2]. Moreover, several inflammatory conditions and autoimmune diseases have been correlated with AQPs dysfunction, although their precise role in the immune system and inflammatory processes is still unclear.

We have recently disclosed the potent and selective inhibition of human AQP3 by gold compounds [3] with no toxic effects to normal cells that would hamper their applicability making them suitable drug leads for *in vivo* studies. In addition, we found that AQP5 is implicated in cell differentiation and is aberrantly expressed in pancreatic tumors of high malignancy, suggesting it may be involved in the inflammatory response and a potential tool for early diagnosis.

Our data clearly suggest AQPs as drug targets and point to the development of aquaporin modulators as therapeutic and diagnostic agents.

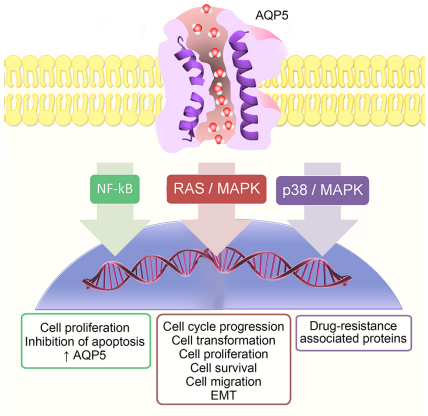

Fig. 1 Schematic representation of AQP5 intracellular signaling pathways involved in cancer [2].

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This abstract fits best into WG2 (diseases) and WG3 (drugs)

Role of the TRPV1 ion channels in anti-tumoral immune response

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There are only few studies examining the role of TRPV1 of immune cells on systemic immune response to cancer. Previous studies suggest that TRPV1 agonists may enhance anti-tumoral immunity.

In this study several different strategies are used to determine the role of TRPV1 channels on anti-tumoral immunity. The liver (4TLM) and brain (4TBM) metastatic cells of murine breast carcinoma which were described by us previously are injected orthotopically to induce tumor formation and lymph nodes and spleens of these tumor bearing animals are used to prepare mix leukocyte cultures (MLC) as part of first strategy. The effects of TRPV1 agonists, antagonists and Gambogic amide which sensitize TRPV1 receptors on cytokine secretion (IFN-γ, IL-12, IL-6, IL-10, TNF-a, IL-1b) from immune cells challenged with LPS and irradiated tumor cells are determined. Our initial experiments demonstrated that TRPV1 agonist alters cytokine response of challenged immune cells (MLC). These effects seemed to be increased in the presence of TRKA agonist Gambogic amide.

In the second part of the study, appropriate TRPV1 agonist / antagonist / Gambogic amide based on previous in-vitro data will be chosen and examine their in-vivo effects on metastasis and systemic immune response in metastatic breast carcinoma. We believe that results of this study will clarify the role of TRPV1 on immune response to cancer, providing novel therapeutic strategies.

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This abstract fits best into: WG1 (cells), WG2 (diseases), WG3 (drugs)

Resting potential of cancer stem cells

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There is a functional relationship between resting membrane potential (Vm) and cell proliferation and differentiation, which can be seen in many cells including normal stem cells and various tumors. Sundelacruz *et al* have shown that Vm depolarization promotes maintenance of mesenchymal stem cells in an undifferentiated stage (1).

We postulated that depolarized Vm may also help maintain a population of cancer stem cells (CSCs), undifferentiated subpopulation of cancer responsible for tumor formation, relapse and metastasis.

Therefore, Vm was measured by Nystatin-perforated patch technique in HMLE cells overexpressing Twist ($HMLE^{twist}$), a model for cancer stem cells (2). Also, we measured Vm of SUM159 and MCF7, two breast cancer cell lines with high and low percentage of CSCs among whole population, respectively.

We show that SUM159 cells are more depolarized than MCF7. Regarding breast CSC model, there was no difference in Vm between $HMLE^{twist}$ and control HMLE cells, pointing to potential limitations of this model.
Furthermore, we used proprietary potassium ionophores (3) to modulate Vm in CSC model. Compound 613 caused depolarization in $HMLE^{twist}$ estimated by using the cationic dye $DioC_6$(3). Preliminary electrophysiological measurements indicate that the compound at first hyperpolarizes cells, which is followed by depolarization.

Measurements of resting potential in CSCs with and without modulators will be further continued. Moreover, we will relate Vm values to expression of various ion channels.

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