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eATP AND P2X RECEPTORS IN INFLAMMATORY CONDITIONS

MURCIA 21ST AND 22ND MARCH 2024

ABSTRACTS BOOK

Fundación para la Formación e Investigación Sanitarias de la Región de Murcia

IMIB Instituto Murciano de Investigación Biosanitaria Pascual Parrilla

UNIVERSIDAD DE MURCIA

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CONTENTS

Thursday 21th March

Room: Sala de Grados (Facultad de Derecho)

08h30 Registration

09h00-09h30 Welcome address session

Prof. Elena Adinolfi	Action Chair
Prof. Pablo Pelegrín	Local Organizer
Dr. Juan José Martínez García	Main Local Organizer
Prof. Pablo Ramírez Romero	Biomedical Research Institute of Murcia (IMIB) Director
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09h30-11h00 Lectures Session 1. Chair Dr. Juan José Martínez-García
Involvement of P2XRs in the molecular mechanisms of inflammation

(10 minutes + 5 minutes questions)

09h:30	Dr. Valentina Vultaggio-Poma (Italy)	The Shed P2X7R (sP2X7R) as useful index in various inflammatory diseases
09h45	Ms. Mª José Caballero Herrero (Spain)	Possible role of P2X7 on NLRP3 inflammasome regulation by Nrf2/DJ-1 pathway stimulation in diabetic nephropathy
10h00	Ms. Adriana Guijarro (Spain)	Plasma membrane permeabilization: roles of Gasdermins and Ninjurin-1
10h15	Dr. Ruth Murrell-Lagnado (UK)	Kv2.1 channels in pain and inflammation
10h30	Dr. Valerie Vouret-Craviari (France)	Activation of the P2RX7/IL-18 pathway in immune cells attenuates lung fibrosis
10h45	Dr. Iva Hafner-Bratkovic (Slovenia)	Agnostic clustering of NLRP3 promotes inflammasome formation

11h00-11h30 Coffee Break

11h30-13h15 Lectures Session 2. Chair Dr. Valerie Vouret-Craviari
P2XRs in cancer and metabolic diseases

(10 minutes + 5 minutes questions)

11h30	Dr. Mohammed Elmallah (France)	Exosomes derived from human MDA-MB-231 breast cancer cells express the P2X4 receptor and induce anti-tumour immune tolerance by decreasing dendritic cell maturation
11h45	Dr. Maria Luiza Thorstenberg (Italy)	Exploration of P2X7R-directed positive allosterism in the generation of anti-cancer response
12h00	Dr. Sahil Adriouch (France)	AAV vector coding for a P2X7-blocking nanobody-based biologic ameliorates colitis
12h15	Ms. Laurenne El Fhaily (France)	Evaluation of nanobody-based biologics targeting purinergic checkpoints in tumour models in vivo

12h30	Prof. Eitan Reuveny (Israel)	Miss regulation of Ca homeostasis by the central control and its effect on sarcopenic obesity phenotype in mice
12h45	Dr. Gennady Yegutkin (Finland)	Tumour cells evade immune responses via modulation of T cell adenosine metabolism and bioenergetics
13h00	Prof. Elena Adinolfi (Italy)	Role of the purinergic adenosinergic axis P2X7/CD39/CD73/A2A in colon carcinoma

13h15-14h45 **Aperitif break**

Room: Hemiciclo (Facultad de Letras)

14h45-15h30 **Round Table Session 1**

Chair Prof. Anna Lisa Giuliani and Dr. Juan José Martinez-Garcia
Does soluble P2XRs have a specific role in inflammation?
(10 minutes introduction + 35 minutes discussion)

15h30-16h15 **Plenary Lecture 1**

Prof. Mathias Chamaillard (France): Diurnal control of immune-mediated protection from metabolic syndrome and infection by the gut microbiota
(35 minutes + 10 minutes questions)

16h15-16h45 **Coffee Break**

16h45-17h30 **Round Table Session 2**

Chair Prof. Pablo Pelegrin and Prof. Luca Antonioli
Does Extracellular ATP measurement a relevant marker in inflammatory diseases?
(10 minutes introduction + 35 minutes discussion)

17h30-17h35 **COST and Tapas. Brief guidance: Dr. Juan José Martínez-García**

17h35-18h35 **Management Committee meeting**

21h00 **Social dinner (optional)**

Friday 22th March

Room: Hemiciclo (Facultad de Letras)

09h15-10h45 **Lectures Session 3. Chair Prof. Carlos Matute**
P2XRs in neuroinflammatory diseases

(10 minutes + 5 minutes questions)

09h15	Mr. Mario Tarantini (Italy)	Measuring extracellular ATP in Alzheimer's disease
09h30	Ms. Andjela Stekic (Serbia)	Neuroinflammation in olfactory bulb is followed by changes in purinergic signalling system in a rat model of multiple sclerosis
09h45	Dr. Milorad Dragic (Serbia)	Prolonged intermittent theta burst stimulation restores the balance between A 2A R- and A 1 R-mediated adenosine signalling in 6-OHDA model of Parkinson´s disease
10h00	Dr. Inés Valencia (Spain)	Pharmacological evaluation of novel non-nucleotide purine derivatives as P2X7 antagonists for the treatment of neuroinflammation in traumatic brain injury
10h15	Ms. Katarina Mihajlovic (Serbia)	Dual CD73/A 2A R inhibition in TNF, IL1-α, C1q-induced neurotoxic astrocytes
10h30	Prof. Nadezda Nedeljkovic (Serbia)	Enhanced CD73/A1R signalling may confer neuroprotection to the cerebellum in experimental autoimmune encephalomyelitis

10h45-11h15 **Coffee Break**

11h15-13h00 **Lectures Session 4. Chair Prof. Jasmina Trojachanec-Pavlovska**
Involvement of P2XRs in physiopathology

(10 minutes + 5 minutes questions)

11h15	Ms. Ana Valeria Vinhais Da Silva (France)	Pathological ventricular remodelling and purinergic signalling: Emerging role of the P2X4 receptor channel
11h30	Dr. Natalia Martínez-Gil (Spain)	Relationship between Purinergic P2X receptors and pannexins in retinal dystrophies
11h45	Dr. Serkan Sen (Turkey)	Targeting P2X receptors in ocular diseases, an example of retinal detachment and dry eye disease
12h00	Ms. Marika Zuanon (UK)	Discovery of new P2X7 Negative Allosteric Modulators for the Treatment of inflammatory Eye Diseases

12h15	Ms. Wiam Echchih (France)	Efficacy of inflammasome inhibition to prevent myocardial ischemia-reperfusion injuries: a protocol for a systematic review and meta-analysis of animal studies
12h30	Prof. Marta Agudo Barriuso (Spain)	Retinal ganglion cell death in a model of sepsis and its rescue by P2X7R antagonism
12h45	Dr. Cécile Delarasse (France)	P2X7 expressed by macrophages controls T-cell mediated autoimmune response

13h00-14h30 **Aperitif break**

14h30-15h15 **Round Table Session 3**

Chair Prof. Dareck Gorecki
Are Inflammatory mediators in the circulation contributing
o neuropsychiatric abnormalities? Case Study: Duchenne Muscular Dystrophy
(10 minutes introduction + 35 minutes discussion)

15h15-16h00 **Plenary Lecture 2**

Prof. Carlos Matute (Spain): Purinergic new avenues in neuroinflammation, tissue damage and repair in neurodegeneration
(35 minutes + 10 minutes questions)

16h00-16h15 **Coffee Break**

16h15-17h00 **Round Table Session 4**

Chair Prof. Elena Adinolfi and Prof. Alison Gartland
Special Issue: Communication and Dissemination
(10 minutes introduction + 35 minutes discussion)

17h00-17h15 **Concluding remarks: Prof. Elena Adinolfi/ Prof. Pablo Pelegrín / Dr. Juan José Martínez García**

PLENARY LECTURES

Diurnal Control of immune-mediated protection from metabolic syndrome and infection by the gut microbiota

Prof. Mathias Chamaillard - Université de Lille, INSERM, Lille, France.

The gut is inhabited by highly diverse communities of microorganisms which are fundamentally adapted to their nutrient-rich environment from the earliest days of life. However, it remains astonishingly unclear how the gut microbiota composition is influenced by daily feeding/fasting cycles for optimal control of weight gain and insulin sensitivity. The disruption of the innate immune system results in an important condition that impedes the glucose homeostasis in the intestinal epithelia, as well as promotes the dysregulation of the intestinal microbiota. The maintenance of this effect longer time can be an important cue that promotes metabolic and inflammatory diseases, such as cancer, type II diabetes, or cardiovascular diseases. As a regulator, feeding behaviour is the animal response to regulate the inflammatory gut conditions and the microbiota composition in a light-dependent manner. While being repressed upon reversal of feeding habits, innate immunity signalling in intestinal epithelial cells impairs cholesterol metabolism promoting aberrant daily cyclic expansion of bile acid-resistant pathobionts during the active phase.

Purinergic new avenues in neuroinflammation, tissue damage and repair in neurodegeneration

Prof. Carlos Matute - Achucarro Basque Center for Neuroscience, University of the Basque Country and CIBERNED, Leioa, Spain.

The biological significance of purinergic receptors in reciprocal neuron-to-glia signaling is still limited. P2X4 and P2X7 receptors are expressed in all brain cells including microglia and oligodendrocytes, two cellular targets along with neurons key in neurodegenerative diseases. We observed that P2X7 receptors mediate excitotoxity both in neurons and in oligodendrocytes in experimental models of stroke and multiple sclerosis. Notably, P2X7 receptor blockade ameliorates symptoms and diminishes tissue damage in these two conditions. These findings point to P2X7 receptors as a key target for neuro- and oligodendrocyte-protection. In turn, we also found that P2X4 receptors control the fate and survival of activated microglia and their phenotype in response to injury in multiple sclerosis and Parkinson´s disease experimental settings. Thus, P2X4 receptor levels and function are upregulated in activated microglia in acute inflammation in those disease paradigms. Importantly, enhanced P2X4 receptor signaling in activated microglia by the positive allosteric modulator ivermectin promotes a regenerative phenotype of microglia that contributes to amelioration of symptoms and neuro- plus oligodendrocyte-protection and repair.

Together, these findings indicate that targeting separately or simultaneously P2X4 and P2X7 receptors may greatly improve tissue protection and repair. Digging into the neurobiology and pharmacology behind these receptors may offer new purinergic avenues to treat neurodegenerative diseases.

Supported by ARSEP, Spanish Ministry of Education, Basque Government and CIBERNED.

LECTURES SESSION 1:
INVOLVEMENT of P2XRs IN THE MOLECULAR MECHANISM OF INFLAMMATION

The shed P2X7R (sP2X7R) as a useful index in various inflammatory diseases

Valentina Vultaggio-Poma[1], Juana Sanz Molina[2], Simonetta Falzoni[1], Angelina Passaro[3], Pantaleo Greco[1], Sara Ghisellini[4], Carla Enrica Gallenga[5], Paola Pizzo[6], Francesco Di Virgilio[1], Anna Lisa Giuliani[1]

[1]Department of Medical Sciences, [2],Department of Chemical, Pharmaceutical and Agricultural Sciences, and [3]Department of Translational Medicine, University of Ferrara, Ferrara, Italy. [4]Laboratory Division and [5]Ophthalmic Clinic Unit, Sant'Anna Hospital of Ferrara, Ferrara, Italy.

Department of Biomedical Sciences, University of Padua, Padua, Italy.

The P2X7R, the preferential receptor for extracellular ATP, is a potent stimulant for NLRP3 inflammasome activation and cytokine release and participates in the pathogenesis of different inflammatory states and diseases. Among others, the P2X7R/NLRP3 pathway has been implicated in various infective, cardiovascular, metabolic, neurologic, and cancer-associated inflammatory conditions. A soluble circulating form of P2X7R (sP2X7R) has been recently found elevated in some inflammatory diseases and tentatively added to the panoply of inflammatory mediators. To this end, we firstly measured circulating levels of sP2X7R in healthy subjects of various ages to better establish reference values. Subsequently, we quantified sP2X7R in different pathologic conditions such as COVID-19 patients, Alzheimer's patients, pregnant women across the three trimesters of pregnancy, and patients with ocular diseases. The examination of sera samples from patients with confirmed SARS-CoV-2 infection with various degrees of disease severity showed that most patients had increased inflammatory and coagulative indexes, and augmented sP2X7R, sNLRP3, IL-6, and IL-10 sera levels. Notably, sP2X7R significantly correlated with inflammatory markers, disease severity and adverse clinical outcome. Data from a limited number of Alzheimer's' patients showed decreased sP2X7R levels respect to age-matched healthy subjects, although increased concentrations were present in subjects with minimal cognitive impairment. Investigation on a higher number of patients will give further information on this regard. Preliminary analysis of maternal sera samples showed that sP2X7R levels were: i) significantly higher in pregnant women at the first trimester of pregnancy than in control age-matched women; ii) increasing across the three trimesters of pregnancy; iii) elevated in early pregnancy in overweight and obese women compared with those with normal BMI. sP2X7R was also detectable in aqueous humour and vitreous humour. We compared sP2X7R levels in healthy conditions and in various ocular or systemic pathological conditions with ocular inflammatory involvement, such as: glaucoma, Fuchs endothelial dystrophy, pseudoexfoliatio, AMD, diabetes mellitus and retinal detachment. Preliminary data from the whole pathologic group compared to control subjects suggest the sP2X7R as a marker of ocular inflammatory status. In conclusion, our observations suggest that the P2X7R soluble form, sP2X7R, might have a role as pro-inflammatory mediator with a function in disease spreading and amplification, and it might also be a useful biomarker in diagnostic medicine.

Further research is nevertheless necessary to define better these results.NED.

Possible Role of P2X7 on NLRP3 inflammasome regulation by Nrf2/DJ-1 pathway stimulation in diabetic nephropathy

María José Caballero-Herrero[1], Laura Hurtado[1], Cristina Molina[1], Celisa Arias-Sanchez[2], Antonio Perez-Olmos[2], Esther Jumilla[1], Pablo Pelegrin[2], Julieta Schachter[1], Santiago Cuevas[1]

[1]BioMedical Research Institute of Murcia (IMIB-Arrixaca), Murcia, Spain.
[2]Immunology department, University of Murcia, Murcia, Spain.

Background: The inflammasome is an important regulator of the inflammatory response and a key factor in the pathogenesis of kidney disease. The renal DJ-1 protein exhibits antioxidant and anti-inflammatory properties and is involved in the regulation of the antioxidant transcription factor Nrf2. To explore novel pharmacological applications of the Nrf2/DJ-1 pathway, we designed ND-13, a peptide consisting of 13 highly conserved amino acids from the DJ-1 sequence.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from diabetic nephropathy patients and controls, plated, and stimulated with LPS+ATP. Mouse bone marrow macrophages (BMDMs) were treated with ND-13 under oxidative stress conditions. Diabetes was induced in C57Bl/6 mice by injection of streptozotocin (STZ) and treated with ND-13 or MCC950, a specific NLRP3 inflammasome inhibitor. The expression of several genes related to inflammation, fibrosis and the NLRP3 inflammasome was analysed by qPCR in peritoneal cells and renal cortex.

Results: PBMCs from patients with diabetic nephropathy treated with LPS+ATP showed a tendency to increase IL-1β release compared to non-diabetic controls, suggesting a potential role of NLRP3 inflammasome in diabetic nephropathy pathogenesis. IL 1β levels in BMDMs medium increased after NLRP3 inflammasome stimulation with LPS+ATP and were significantly decreased after ND-13 pretreatment in the presence of H_2O_2 (100 nM). STZ-induced diabetes in mice significantly increased the mRNA expression of COL-I, COL-II, TGF-β, IL-6, TNF-α and P2X7 in the renal cortex, which was partially prevented by ND-13 and pretreatment with MCC950 (a NLRP3 inflammasome inhibitor). IL-1β release by peritoneal macrophages obtained from diabetic mice was increased after cell stimulation with LPS+ATP, comparing with non-diabetic mice, suggesting that the inflammasome is activated in diabetes, and ND-13 treatment normalised its activity. P2X7 mRNA expression was also increased in diabetic peritoneal macrophages, which expression was attenuated by ND-13 pre-treatment.

Conclusion: ND-13 may be a novel pharmacological approach to attenuate the deleterious effects associated with inflammasome activation in renal disease. Furthermore, our data suggest that P2X7 may be involved in the molecular mechanism by which ND-13 exerts such a protective role by inhibiting inflammasome activation in diabetic nephropathy.

Plasma membrane permeabilization: roles of Gasdermins and Ninjurin-1

Adriana Guijarro, Pablo Pelegrín, Julieta Schachter

BioMedical Research Institute of Murcia (IMIB-Arrixaca), Murcia, Spain.

Pyroptosis is a highly pro-inflammatory type of lytic cell death. Among the mediating proteins in this cell death process, gasdermins (GSDMs) stand out, a family of proteins made up of six members in humans (GSDMA, GSDMB, GSDMC, GSDMD, GSDME and PJVK), present in different cell types and tissues with differential expression. Structurally, these proteins show a C-terminal repressive domain and another cytotoxic or N-terminal domain linked by a linker region where they can undergo proteolytic cleavage by different proteases, thus releasing the N-terminal domain. This domain binds to membrane lipids, oligomerizing and forming pores that permeabilize the membrane, through which intracellular proteins can be released, including the active form of proinflammatory cytokines such as IL-1β or IL-18. If the pores of GSDMs are not resolved, the plasma membrane may eventually lose its integrity due to the oligomerization of the transmembrane protein ninjurin 1 (NINJ1), resulting in the release of more and larger intracellular content, such as lactate dehydrogenase (LDH), among many other components. However, the mechanisms which lead to the activation of NINJ1 and induction of pyroptosis after the formation of GSDM pores are currently unknown. On the other hand, it is known that intracellular ATP can be released by cells through lytic and non-lytic processes, but the mechanisms involved in this release are still matter of debate. The cytoprotective effect of the amino acid glycine has long been known, but only recently was it shown that glycine impairs the oligomerization of NINJ1 on the cellular membrane. Nevertheless, it is not yet known what the molecular mechanism is responsible for lytic prevention by glycine. In this study we focus on characterizing the possible effects of glycine on the membrane permeabilization induced by the different GSDMs and NINJ1. We evaluated the release of LDH and ATP, as well as YO-PRO-1 uptake, in an attempt to separate the different phenomena related to the formation of GSDMs and NINJ1 pores. This knowledge is fundamental for the understanding of the inflammatory pyroptotic death and could reveal new therapeutic strategies for the treatment of different diseases with inflammatory components through pharmacological modulation of GSDMs and NINJ1 proteins.

Kv2.1 channels in pain and inflammation

Ruth Murrell-Lagnado

Sussex Neuroscience, School of Life Sciences, University of Sussex, Brighton, UK

Kv2.1 channels give rise to high-threshold, slowly activating outward K+ currents in a wide-range of neurons in both the central and peripheral nervous systems (CNS, PNS). They play a particularly important role in suppressing elevated neuronal firing and compromising their function in sensory neurons leads to hyperexcitability associated with chronic pain. Elevated function is also detrimental, being responsible for the pro-apoptotic K+ efflux in CNS neurons that is observed following brain ischemia.

The Kv2.1 channel has intriguing properties in that it features unusual subcellular localisation in the form of micrometre size plasma membrane clusters, regulation by several 'silent' Kv subunits and important non-conducting functions. The clusters comprise non-conducting channels that stabilize junctions between the endoplasmic reticulum and PM and act as trafficking hubs for recruiting other ion channels and controlling Ca2+ homeostasis and vesicle exocytosis.

Our research has focused on understanding the impact of one of the 'silent' Kv subunit (Kv9.1) on Kv2 channel cluster formation with the aim to understand the link between SNPs in the Kv9.1 gene and development of chronic pain. In addition, we have identified novel, peripherally restricted activators of Kv2.1 channels with the aim to interrogate the channel as a target for novel analgesics.

Activation of the P2RX7/IL-18 pathway in immune cells attenuates lung fibrosis

Valerie Vouret-Craviari

Université Côte d'Azur, CNRS, INSERM, IRCAN, Nice, France.

Idiopathic pulmonary fibrosis (IPF) is an aggressive interstitial lung disease associated with progressive and irreversible deterioration of respiratory functions that lacks curative therapies. Despite IPF being associated with a dysregulated immune response, current antifibrotics aim only at limiting fibroproliferation. Transcriptomic analyses show that the P2RX7/IL18/IFNG axis is downregulated in IPF patients and that P2RX7 has immunoregulatory functions. Using our positive modulator of P2RX7, we show that activation of the P2RX7/IL-18 axis in immune cells limits lung fibrosis progression in a mouse model by favoring an anti-fibrotic immune environment, with notably an enhanced IL-18-dependent IFN-γ production by lung T cells leading to a decreased production of IL-17 and TGFβ. Overall, we show the ability of the immune system to limit lung fibrosis progression by targeting the immunomodulator P2RX7. Hence, treatment with a small activator of P2RX7 may represent a promising strategy to help patients with lung fibrosis.

Agnostic clustering of NLRP3 promotes inflammasome formation

Elvira Boršić[1,2], Sara Orehek[1,2], Taja Železnik Ramuta[1], Mateja Erdani Kreft[3] and Iva Hafner-Bratkovič[1,3,4]

[1]Department of Synthetic Biology and Immunology, National Institute of Chemistry, Ljubljana, Slovenia
[2]Interdisciplinary Doctoral Study of Biomedicine, Medical Faculty, University of Ljubljana, Ljubljana, Slovenia
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[4]EN-FIST Centre of Excellence, Ljubljana, Slovenia

NLRP3 inflammasome assembles in response to versatile activators and is involved in a variety of pathological conditions. There has been significant progress in the determination of the structural mechanism of NLRP3 inflammasome assembly from inactive LRR domain-facilitated cages to active NACHT domain-based discs. However, it is not clear how different triggers and organelles facilitate NLRP3 inflammasome assembly. We performed an extensive synthetic biology study, where engineered NLRP3 variants that are enriched at different subcellular locations were introduced into NLRP3-deficient macrophages. We show that NLRP3 variants enriched at Endoplasmic reticulum, Golgi apparatus, lysosomes, mitochondria, plasma membrane and peroxisomes respond to canonical NLRP3 triggers with ASC speck formation, cell death and proinflammatory cytokine IL-1β secretion similarly to wild-type NLRP3. In contrast to wild-type NLRP3 that is nonfunctional, the deletion of a positively charged cluster did not affect the activation of localized variants. While the majority of organelle-enriched NLRP3 variants were not active in the absence of the triggers, simultaneous clustering of NLRP3 led to constitutive activation that did not originate in enforced oligomerization of the pyrin domain but rather in the loosening of the inactive NLRP3 conformation. Our results show that NLRP3 can be activated from various subcellular locations and emphasize the need for NLRP3 clustering that can be achieved through binding to lipid or protein scaffolds. Our results argue against uniform NLRP3 activation pathway but suggest that versatile NLRP3 triggers could engage different activation pathways as long as they induce NLRP3 clustering that facilitates NLRP3 inflammasome assembly.

LECTURES SESSION 2:
P2XRs IN CANCER AND METABOLIC DISEASES

Exosomes derived from human MDA-MB-231 breast cancer cells express the P2X4 receptor and induce anti-tumor immune tolerance by decreasing dendritic cell maturation

Mohammed Elmallah[1], Audrey Heraud[1], Thomas Duret[1], Christophe Baron[1], and Sébastien Roger[1]

[1]INSERM U1327 ISCHEMIA "Membrane Signaling and Inflammation in reperfusion injuries", University of Tours, Tours, France

Exosomes are small extracellular nano-vesicles bilayer (50-200nm), produced by all cell types within the endosomal compartment. Exosomes contain proteins, RNAs and other substances that can be transferred to neighboring or distant cells, affecting their behaviors. The interaction between tumor and tumor microenvironment (TME)-associated players can facilitate tumor escape from immune surveillance and promote cancer progression as well. Dendritic cells (DCs) are suggested to play an essential role in the anti-tumor immune surveillance due to its antigen presenting ability and subsequent activation of T-cells-mediated response. However, a growing evidence suggests that tumor-derived exosomes (TDE) can regulate both the phenotype and the function of DCs, as well as other immune cells, thus inducing immune tolerance conditions against tumor. Recently, we demonstrated that the P2X4 receptor modulates the production of exosomes by breast cancer cells, and that cancer exosomes express this receptor.

Therefore, the current study tends to investigate the effect of human MDA-MB-231 breast cancer cell-derived exosomes, expressing or not P2X4, on the immune modulation of DCs. Here, we show that treatment of immature DCs with. MDA-MB-231-exosomes that express P2X4 displayed a significant reduction in the surface expression of both differentiation and maturation markers of DCs including CD209, CD83, CD86 and CD25 compared to both non-treated cells and those stimulated with LPS. To investigate the effect of P2X4 on the modulation of DCs, we generated a stable P2X4 knockout MDA-MB-231 cell line (MDA-MB-231 -/-P2X4) and the immunomodulation effect of their derived exosomes on DCs will be studied. In conclusion, our preliminary data suggest the implication of P2X4 in the induction of DCs immune tolerogenic phenotype against BC tumor.

Exploration of P2X7R-directed positive allosterism in the generation of anti-cancer response

[1]Maria Luiza, Thorstenberg,[1]Edith, Garcia-Jacobo., [1]Valentina, Vultaggio-Poma.,[1]Leticia Scussel Bergamin.,[1]Simoneta, Falzoni and, [1]Francesco, Di Virgilio.

[1]Department of Medical Sciences, University of Ferrara, Ferrara, Italy.

Nucleotides, mainly extracellular ATP can be release into the TME by both tumour and host cells. P2X7R has been assigned multiple and often contrasting roles as a driver of cancer cell growth and as a promoter of immune-mediated tumor erradication. Interestingly, approaches based on either P2X7R antagonism or agonism have been shown to be effects in reducing tumor growth, thus leaving many open questions on the mechanisms underlying P2X7 activity in cancer. Here, we explored the anti-tumor response mediated by the treatment of two positive allosteric modulators of the P2X7R, as well: Clemastine fumarate (CLE) and Polymyxin B (PB) in melanoma tumor bearing mice. We evaluated the ethidium bromide permeability and intracellular Ca2+ level i[ca2+] in B16F10WT cells, pulsed or not with CLE. Cells were treated with Bz-ATP and P2X7 blockers to analyze P2X7 functions. The data were expressed as % permeabilization and i[ca2+] respectively. We measured IL-1β secretion in macrophages, 2,0x105 perithoneal macrophages from C57Bl/6 mice were plateled by 24h and immediately primed with LPS (1µg/mL) for 2 h. The culture received CLE in combination with 3mM ATP or not, for 30 min, then supernatant were harvested and ELISA was performed to quantificate IL-1β release. Next, we evaluated CLE/PB effects in tumor bearing mice. Male 4- to 8-week-old C57Bl6 WT or C57BL6 P2X7-/- mice were inoculated subcutaneously with 2.0 x 105 B16F10. 5 d.p.i we started the treatment with P2X7 allosteric drugs (CLE 10mg/kg and PB 5mg/Kg). Tumor size was measured with a manual caliber, and tumor volume was calculated as described. Mice received five treatments at interval range 2 to 3 days, 24 hours post the last treatment we performed the euthanasia and tumor were harvested to evaluation of the positive proliferating cells (mAb Ki-67 staining), immune infiltrate (mAb CD-3, CD4, CD8, F4/80 staining) in spleen and exicised tumor by IHC.

We measured P2X7 functionality mediated by positive alosterism, and our data demonstrated that the combined stimulation with CLE and Bz-ATP increased pore-forming (EB uptake) function and channel i[ca2+] in B16F10 cells, when compared with Bz-ATP treatment. In contrast, the P2X7R antagonist A740003 abrogated this effect. These data suggest that CLE sensitizes cells to Bz-ATP, probably by direct interaction with P2X7. However, CLE or PB treatment enhanced growth of B16F10 melanoma cells inoculated in C57Bl/6 mice. Interestingly, the tumor growth effect was enhanced in C57Bl/6 P2X7-/- mice. The association of the P2X7R with inflammation and immunity is long standing. Immune cells express this receptor to a high level and P2X7 triggered effector mechanism in the immune system. In accordance, WT mice that received CLE enhanced the % CD3, CD4 and CD8 lymphocytes from spleen and tumor. Moreover, CLE treatment enhanced ATP-dependent IL-1β secretion from WT peritoneal macrophages. Thus, it is likely that P2X7R allosterism in vivo affects both host immune cells and tumor cell responses (by potentiate tumor growth), the present result being the combination of both effects. We observed that in vivo, CLE induced typical responses of the P2X7 receptor in the context of TME, resulting in a dual effect both by activating tumors and by activating immune cells.

AAV vector coding for a P2X7-blocking nanobody-based biologic ameliorates colitis

Sahil Adriouch

Faculté de Médecine et de Pharmacie, Inserm - Unité Mixte de Recherche U 1234 (PANTHER), Université de Rouen Normandie Institut de Recherche et d'Innovation Biomédicale (iRiB)

The pro-inflammatory ATP-gated P2X7 receptor is widely expressed by immune and non-immune cells. Nanobodies targeting P2X7, with potentiating or antagonistic effects, have been developed and adeno-associated virus (AAV)-mediated gene transfer represents an efficient approach to achieve long-term in vivo expression of selected nanobody-based biologics. We aimed here to use this approach (termed AAVnano) to validate the relevance of P2X7 as a target in dextran sodium sulfate (DSS)-induced colitis in mice. For that, mice received an intramuscular injection of AAV vectors coding for potentiating (14D5-dimHLE) or antagonistic (13A7-Fc) nanobody-based biologics targeting P2X7. Long-term modulation of P2X7 activity was evaluated ex vivo from blood samples. Colitis was induced with DSS in mice injected with AAV vectors coding for nanobody-based biologics. Severity of colitis, colon histopathology and expression of chemokines and cytokines were determined to evaluate the impact of P2X7 modulation. A single injection of an AAV vector coding for 13A7-Fc or 14D5-dimHLE efficiently modulated P2X7 function in vivo from day 15 up to day 120 post-injection in a dose-dependent manner. An AAV vector coding for 13A7-Fc significantly ameliorated DSS-induced colitis and significantly reduced immune cell infiltration and expression of chemokines and proinflammatory cytokines in colonic tissue. In conclusion, we have demonstrated the validity of AAVnano methodology to modulate P2X7 functions in vivo. Applying this methodological approach to a DSS-induced colitis model, we have shown that P2X7 blockade reduces inflammation and disease severity. Hence, this study confirms the importance of P2X7 as a pharmacological target and suggests the use of nanobody-based biologics as potential therapeutics in inflammatory bowel disease.

Evaluation of nanobody-based biologics targeting purinergic checkpoints in tumor models in vivo

Laurenne El-Fhaily, Sahil Adriouch

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Adenosine triphosphate (ATP) represents a danger signal that accumulates in injured tissues, in inflammatory sites, and in the tumor microenvironment. ATP promotes tumor growth but also anti-tumor immune responses notably via the P2X7 receptor. ATP can also be catabolized by CD39 and CD73 ecto-enzymes into immunosuppressive adenosine. P2X7, CD39 and CD73 have attracted much interest in cancer as targets offering the potential to unleash anti-tumor immune responses. These membrane proteins represent novel purinergic checkpoints that can be targeted by small drugs or biologics. We investigated nanobody-based biologics targeting mainly P2X7, but also CD73, alone or in combination therapies. Blocking P2X7 inhibited tumor growth and improved survival of mice in cancer models that express P2X7. P2X7-potentiation by a nanobody-based biologic was not effective alone to control tumor growth however enhanced tumor control and immune responses when used in combination with oxaliplatin chemotherapy. We also evaluated a bi-specific nanobody-based biologic that targets PD-L1 and CD73. This novel nanobody-based biologic exerted a potent anti-tumor effect, promoting tumor rejection and improving survival of mice in two tumor models. Hence, this study highlights the importance of purinergic checkpoints in tumor control and open new avenues for nanobody-based biologics that may be further exploited in the treatment of cancer. Also, this study pave the way for the development of novel bi-specific nanobodies targeting several purinergic checkpoints (such as CD73 and CD39) or combining one purinergic checkpoint and one classical immune checkpoint (such as PD-1/PD-L1).

Miss regulation of Ca homeostasis by the central control and its effect on sarcopenic obesity phenotype in mice

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Store-Operated Calcium Entry (SOCE) is a vital process aimed at refilling cellular internal Ca2+ stores and a primary cellular-signaling driver for transcription factors' entry to the nucleus. SARAF (SOCE-associated regulatory factor)/TMEM66 is an endoplasmic reticulum (ER) resident transmembrane protein that promotes SOCE inactivation and prevents Ca2+ overfilling of the cell. Here we demonstrate that mice deficient in SARAF develop age-dependent sarcopenic obesity with decreased energy expenditure, lean mass, and locomotion without affecting food consumption. Moreover, SARAF ablation reduces hippocampal proliferation, modulates the activity of the hypothalamus-pituitary-adrenal (HPA) axis, and mediates changes in anxiety-related behaviors. Interestingly, selective SARAF ablation in the hypothalamus's paraventricular nucleus (PVN) neurons reduces old age-induced obesity and preserves locomotor activity, lean mass, and energy expenditure, suggesting a possible central control with a site-specific role for SARAF. At the cellular level, SARAF ablation in hepatocytes leads to elevated SOCE, elevated vasopressin-induced Ca2+ oscillations, and an increased mitochondrial spare respiratory capacity, thus providing insights into the cellular mechanisms that may affect the global phenotypes. These effects may be mediated via the liver X receptor (LXR), and IL-1 signaling metabolic regulators explicitly altered in SARAF ablated cells. In short, our work supports both central and peripheral roles of SARAF in regulating metabolic, behavioral, and cellular responses.

Tumour cells evade immune responses via modulation of T cell adenosine metabolism and bioenergetics

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Despite the success of immune checkpoint therapies such as anti-PD1 and anti-CTLA4, there are many patients who do not benefit from these treatments. One promising target for addressing immune checkpoint therapy-resistant cancer is adenosine pathway. High levels of adenosine in the tumour microenvironment suppress the proliferation, activation, and effector functions of T-cells through adenosine receptor-mediated mechanisms, allowing tumours to evade the immune response. We hypothesised that cancer cells can directly manipulate the purine metabolism of T-cells, thereby promoting a less reactive T-cell phenotype. In our study, human peripheral blood T-cells were co-cultured with human breast cancer cells MDA-MB-231 in the absence and presence of anti-CD3/CD28 beads, and subsequently analysed by flow cytometry and confocal microscopy. Our results reveal selective up-regulation of the main nucleotide-inactivating enzymes CD39 and CD73 on the activated T-cells upon co-culture with cancer cells, which requires direct cell-cell contact and is presumably mediated through intercellular nanotubes. Furthermore, our findings indicate that high micromolar concentrations of adenosine suppress bioenergetics of activated T-cells and promote their apoptosis via cellular adenosine uptake through the equilibrative nucleoside transporters, independently of adenosine receptor activation. Taken together, these data suggest novel mechanisms by which cancer cells create adenosine-rich environment and dampen T-cell responses.

Role of the purinergic adenosinergic axis P2X7/CD39/CD73/A2A in colon carcinoma

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Colon carcinoma is one of the most diffused cancers in the world, and although it is often curable in its early stages, metastatic forms remain a clinical challenge. Interestingly, colon carcinoma development is connected with inflammatory reactions. Therefore, the purinergic adenosinergic axis that is associated with both inflammatory and anti-inflammatory responses and carcinoma progression is a natural pathway to be investigated in colon carcinoma. Here, we analyzed the expression of P2X7, CD39, CD73, and A2A in colon carcinoma patients and different murine models. All of the purinergic adenosinergic axis components were upregulated in the late stages of colon carcinoma, especially in metastasis-derived specimens.

Moreover, we analyzed by immunohistochemistry the expression of these molecules in Pirc rats, which carry a mutation in the Apc gene leading to the spontaneous development of colon cancer, demonstrating that P2X7, CD73, CD39, and A2A were all increased in the either in the colon of the mutant rats or in spontaneous tumors developing within the intestinal tract. Finally, we tested P2X7 and A2A antagonists in mice models of primary and metastatic colon carcinoma, demonstrating their efficacy in reducing colon carcinoma growth and dissemination alone or in combination.

Our data confirm the interplay between different components of the purinergic adenosinergic axis in colon carcinoma and suggest that targeting multiple components of this pathway could be a novel therapeutic approach to treat advanced forms of cancer.

LECTURES SESSION 3:
P2XRs IN NEUROINFLAMMATION

Measuring extracellular ATP in Alzheimer's disease

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Background: Alzheimer's disease (AD) is a devastating neurodegenerative disorder currently affecting over 47 million people worldwide, expecting to rise to more than 131 million by 2050. AD is characterized by progressive cognitive decline accompanied with accumulation of aberrant proteins such as hyperphosphorylated tau protein and amyloid β (Aβ) peptides, forming neurofibrillary tangles and senile plaques, respectively. (Breijyeh Z, et al. Molecules. 2020 Dec 8;25(24):5789). Despite over a century of investigation, etiology and pathogenesis of AD are still unclear, albeit growing evidence supports a central role of neuroinflammation. A major role in inflammation is played by extracellular ATP (eATP), which is a versatile immunomodulatory agent and pro-inflammatory molecule acting at P2Y and P2X purinergic receptors, with the P2X7 receptor, P2X7R, subtype being the most frequently involved (Sanz JM, J Immunol. 2009 Apr 1;182(7):4378-85). Altered expression levels and function of P2X7R were found both in AD patients and AD mouse models. (Sanz JM, et al. Exp Gerontol. 2014 Dec;60:117-9). Accordingly, genetic depletion or pharmacological inhibition of P2X7R ameliorated the hallmarks and symptoms of different AD mouse models. While being in the low nanomolar range in healthy tissues, the eATP concentration ([eATP]) can reach tens or even hundreds of micromoles/L at sites of trauma or inflammation, AD brain included. (Francistiová L, et al. Front Mol Neurosci. 2020 Jun 3;13:94).

Aim: Several studies have identified the importance of purinergic signaling in AD, nevertheless, extracellular ATP levels have never been measured in AD brain. In order to fully comprehend the functioning of purinergic receptors in AD, we are trying to set up a new system to track eATP variation in vivo. Therefore, the specific aim of our work is to develop a new ratiometric probe, based on a dual-Luciferase system, to detect changes in [eATP] in healthy and AD brain.

Experimental approach

The new ratiometric probe was validated by engineering three stably transfected cell lines: Human Embryoinic Kidney (HEK293) cells, murine microglia (N13) cells, murine melanoma (B16) and murine colon carcinoma (CT26). The enzymatic activity was assessed in vitro as well as in luciferin-injected mouse models by using IVIS luminometer and VICTOR luminomiter (PerkinElmer).

Key results: Our results show that luminescence emission increases with [eATP] in vitro. Furthermore, this ratiometric probe allows normalization of the luminescence signal to the cell number, and therefore accurate measurement of [eATP]. In vivo preliminary data show that this dual luciferase probe allows measurement of [eATP] in the tumor microenvironment of B1610F melanoma tumors, and is sensitive to ATP release triggered by an inflammatory stimulus.

Conclusions: These data show that changes in [eATP] can be accurately monitored by using the dual luciferase probe. This is a substantial advancement over the canonical pmeLUC sensor originally developed by our laboratory (Pellegatti P, et al. Mol Biol Cell. 2005 Aug;16(8):3659-65; Pellegatti P et al. PLoS One. 2008 Jul 9;3(7):e2599), and hopefully a useful tool for the investigation of AD and other neurodegenerative diseases.

Neuroinflammation in olfactory bulb is followed by changes in purinergic signaling system in a rat model of multiple sclerosis

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Olfactory dysfunction is a common symptom in neurodegenerative disorders, including Parkinson's and Alzheimer's disease, which appear decades before motor disability and cognitive decline. Clinical studies have shown that a certain percentage of multiple sclerosis (MS) patients manifest reduced olfaction in the prodromal phase of the disease. The underlying mechanism of olfactory dysfunction and the changes in the olfactory pathway may be central to understanding the autoimmune disease onset and progression. The aim of this study was to examine olfactory dysfunction-related neuroinflammation and potential changes in purinergic signaling system in olfactory bulb of rats suffering from experimental autoimmune encephalomyelitis (EAE), a common animal model of MS. Male 2-month-old Dark Agouti (DA) rats were subjected to immunization with spinal cord homogenate in complete Freund's adjuvant enriched with Mycobacterium tuberculosis. Rats were scored daily for EAE symptoms and sacfriced in the paralytic stage of the disease (11-13 dpi) to isolate olfactory bulb tissues. Thereafter, tissues were used for immunohistochemical, qPCR and immunoblot analyses. Immunohistochemical staining showed prominent gliosis expressed as increased GFAP and Iba1 immunoreactivity in EAE animals; qPCR exhibited upregulation of Entpd1, Nt5e, Adora3, P2rx4, P2ry12, downregulation of Entpd2, whereas gene expression of Ada, Ent1, Ent2, Adora1, Adora2a, Adora2b, P2rx2, P2rx7 and P2ry1 remained at the control level. Protein expression of eN/CD73, A1R, A2BR, A3R and P2Y1R was elevated in EAE, as opposed to P2X7R and P2Y12R that showed a decrease in protein expression. Nevertheless, protein expression of A2AR and ADA did not show a statistically significant change. Since purinergic signaling plays a pivotal role in odor processing in olfactory bulb, as well as in inflammatory responses, results in this research field could lead to better understanding of olfactory dysfunction in MS/EAE, which could be used as a potential diagnostic tool. The meaning and significance of described changes in purinergic signaling are yet to be elucidated.

Prolonged intermittent theta burst stimulation restores the balance between A2AR- and A1R-mediated adenosine signaling in 6-OHDA model of Parkinson's disease

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An imbalance in adenosine-mediated signaling, particularly increased A2AR-mediated signaling, plays a role in the pathogenesis of Parkinson's disease (PD). Existing therapeutic approaches fail to alter disease progression, demonstrating the need for novel approaches in PD. Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive approach shown to improve motor and /nonmotor symptoms of PD. However, the underlying mechanisms of the beneficial effects of rTMS are unknown. To investigate the extent to which the beneficial effects of prolonged intermittent theta burst stimulation (iTBS) in the 6-hydroxydopamine (6-OHDA) model of experimental parkinsonism is based on modulation of adenosine-mediated signaling. Animals with unilateral 6-OHDA lesions underwent iTBS for three weeks and tested for motor skills in the rotarod test. Immunoblot, qRT-PCR, immunohistochemistry, and biochemical analyzes of component of adenosine-mediated signaling were performed on synaptosomal fraction of the lesioned caudoputamen (CPu). Prolonged iTBS improved motor symptoms in 6-OHDA-lesioned animals. 6-OHDA lesion resulted in progressive loss of dopaminergic neurons in CPu. Treatment with iTBS began 7 days after lesion with the onset of motor symptoms. After prolonged iTBS treatment, complete motor recovery was observed. This improvement was accompanied by downregulation of the eN/CD73-A2AR and a return to physiological levels of A1R-ADA1 after 3 weeks of iTBS. Our results showed that 6-OHDA-induced degeneration minimizes the expression of A1R and increase the expression of A2AR. iTBS counteracted these effects by restoring the abundance of A1R and A2AR to control levels. The shift in ARs expression most likely restored the balance between dopamine-adenosine signaling, leading to recovery of motor control.

Pharmacological evaluation of novel non-nucleotide purine derivatives as P2X7 antagonists for the treatment of neuroinflammation in traumatic brain injury

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Traumatic brain injury (TBI) is an acute brain lesion acknowledged as one of the main causes of mortality and disability worldwide. Brain injury after TBI is spread in the long term, with neuroinflammation playing a key role in the development of secondary brain sequelae. Searching for therapeutic strategies that shrink the inflammatory response after TBI is key to prevent and/or ameliorate secondary brain injury and to improve the outcome of patients. After TBI there is a fast activation of innate immunity, where microglia is activated in response to damage-associated molecular patterns such as ATP, which is detected by P2X7 purinergic receptors. Indeed, the P2X7-NLRP3 inflammasome axis has been identified as one of the main actors in neuroinflammation. Thus, the aim of this study was to validate P2X7 as a potential therapeutic target in TBI, as well as to evaluate new non-nucleotide purine derivative compounds as P2X7 antagonists in models of TBI both in vitro and in vivo.

First, serum P2X7 levels were evaluated in patients after TBI, observing a significant reduction of P2X7 after 72h of brain lesion. P2X7 was then validated as pharmacological target by genetic inhibition, performing the closed head injury model of TBI in p2x7-deficient mice. p2x7 -/- mice showed worse transcriptomic proinflammatory profile at brain level as well as poorer neuroconductual score 24h after TBI, in comparison to control mice. However, the animals that were treated with the potent and selective P2X7 antagonist JNJ-47965567 (30 mg/kg i.p.) 30 minutes after TBI, presented a tendency towards improvement of their neurological and inflammatory parameters. These apparently differing results depicted the relevance of P2X7 activation in a time-dependent control of traumatic lesion, which was further evaluated in a second pharmacological approach. A series of purine derivative compounds, divided into etanoil and sulfonil families according to their chemical structure, were evaluated as P2X7 antagonists in the context of TBI. Those compounds that prevented IL-1β release from primary mixed glial cultures in response to LPS+ATP, were further evaluated in vivo. In the etanoil family, ITH15004 (1 mg/kg i.p.) injected 30 minutes after TBI, improved inflammatory markers 24h after TBI. On the other hand, ITH15003 was the most effective compound from the sulfonil family and it will be shortly evaluated in vivo. Altogether, these results highlight the implication of P2X7 as potential therapeutic target to modulate neuroinflammation course in TBI.

Dual CD73/A2AR inhibition in TNF, IL1-α, C1q-induced neurotoxic astrocytes

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Ecto-5'-nucleotidase (CD73) is a cell adhesion protein and a membrane-bound ecto-phosphohydrolase that hydrolyzes adenosine monophosphate (AMP) to adenosine. Adenosine is an important signaling molecule that acts via one of four subtypes of adenosine receptors (ARs), leading to stimulation (A1R, A3R) or inhibition (A2AR, A2BR) of adenylyl cyclase. In many chronic neurodegenerative diseases, such as multiple sclerosis, Alzheimer's disease and Parkinson's disease, an increase in CD73 activity and expression leads to a higher concentration of adenosine in the extracellular space resulting in excessive activation of A2AR, which triggers proinflammatory signaling pathways that further promote neurodegeneration. Therefore, attenuation of CD73/A2AR signaling is a potential pharmacological target in chronic neurodegeneration. In the present study, we used a novel in vitro model of neuroinflammation induced by activation of primary astrocytes with a combination of proinflammatory cytokines, TNF (30 ng/ml), IL-1α (3 ng/ml), and C1q (400 ng/ml), which together are necessary and sufficient to induce a neurotoxic astrocyte phenotype (TIC -induced reactive astrocytes). In order to examine the efficacy of dual CD73/A2AR blockade, TIC -induced reactive astrocytes were then treated with widely used CD73 inhibitor- adenosine 5'-(α,β-methylene)diphosphate - APCP (100 μmol/l) and A2AR antagonist- istradeffyline (10 μmol/l), which is FDA-approved and used as cotherapy in treatment of Parkinson's disease. Our results show that selected concentrations of APCP and istradeffyline together do not affect cell viability and metabolic activity in primary rat cortical astrocytes. The TIC model was first used to examine the temporal pattern of CD73 and A2AR expression and their membrane localizations in order to provide a suitable model for testing the efficacy of dual CD73/A2AR blockade. Dual CD73/A2AR inhibition by using APCP and istradeffyline in TIC-induced model of reactive astrocyte reduces neuroinflammation on a gene level by inducing a decreasing trend in the expression of Vcam1, marker of TIC-induced reactive astrocytes and proinflammatory genes such as C3 and also reduces parameters of oxidative stress by increasing activity of antioxidative enzymes.

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Enhanced CD73/A1R signaling may confer neuroprotection to the cerebellum in experimental autoimmune encephalomyelitis

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Experimental autoimmune encephalomyelitis (EAE) is a widely recognized animal model for multiple sclerosis (MS). Most EAE studies focus on the spinal cord, as demyelination and axonal degeneration in this part of central nervous system (CNS) are responsible for the apparent neurological symptoms in EAE, while the rest of CNS has been much less studied. However, a systematic study of the whole mouse brain has shown that autoimmune cell infiltration in EAE begins at similar time points through the ependymal wall and choroid plexus and affects many brain areas, most notably the central sensory pathways and their terminal fields, including olfactory, visual, auditory/vestibular, somatosensory (lemniscal) and proprioceptive (spinocerebellar). We examined immune cell infiltration through the choroid plexus at the level of the fourth cerebral ventricle into the adjacent brain regions - pontine tegmentum (PT) and cerebellum (CER), which are the part of the proprioceptive system. There was a striking difference in neuroinflammation in the adjacent areas in EAE. While PT showed numerous infiltrated CD4+ T cells and macrophages, marked gliosis and demyelination, there were no signs of infiltration and neuroinflammation in the CER. Since enhanced CD73/A2AR signaling is required for the opening of the choroid plexus barrier for infiltration of CD4+ T cells, we wanted to investigate adenosinergic signaling at this level. The data show a significant upregulation of the adenosine-producing enzyme CD73 and an unchanged activity of the adenosine-metabolizing enzyme adenosine deaminase (ADA) in both PT and CER. However, the increase in extracellular adenosine occurred in different glial cell types and was associated with different adenosine receptors in the investigated regions. In particular, infiltrated macrophages and ameboid microglia in PT overexpressed excitatory A2AR and A2BR, whereas in the CER, an increase in CD73 occurred together with upregulation of inhibitory A1R, mainly in specialized cerebellar Bergman astroglia. Because A1R and A2AR are functionally antagonistic to each other, the increased adenosine production in EAE and its effect on a specific AR subtype in glial cells and cells that form tissue barriers could represent a mechanistic switch that may alter barrier properties and permeability during EAE. Enhanced CD73-A1R coupling in Bergmann glia may support the barrier properties of CER ependymal cells, while enhanced CD73-A2AR coupling may lead to immune cell infiltration into the surrounding PT during EAE. Interestingly, while several other inflammatory indicators (IL-1β, THF-αa, C3, IL-10, CNPaseII) also showed an opposite trend in PT and CER during EAE, the expression of P2X7R decreased significantly in both studied areas. In conclusion, specialized Bergmann astroglia and the intrinsic differences in adenosinergic signaling may play a role in the differential regional susceptibility to EAE inflammation.

LECTURES SESSION 4:
INVOLVEMENT OF P2XRs IN PHYSIOPATHOLOGY

Pathological ventricular remodeling and purinergic signaling: Emerging role of the P2X4 receptor-channel

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Pathological left ventricular remodeling is a complex process involving the architectural disorganization of cardiac tissue subsequent a myocardial infarction. This phenomenon is characterized by the exaggerated development of fibrotic scar tissue. This non-contractile, poorly conductive tissue can eventually lead to organ dysfunction and heart failure, although the exact factors contributing to this process remain unknown. Current research is dedicated to understanding and modulating the inflammatory response, a key aspect in the process of tissue healing. We hypothesized that an altered response of cardiac fibroblasts, undergoing cellular stress induced by coronary occlusion, might be implicated in the development of pathological post-infarction left ventricular remodeling. This response is orchestrated by molecular switches that allow fibroblasts to sense their environment, survive during cellular stress, and regulate their response when necessary. Among these molecular switches, P2X4 appears to play a pivotal role due to its sensitivity to extracellular ATP, a molecule frequently released during cellular stress. Additionally, P2X4 has been found to be involved in the regulation of autophagy, a critical pathway for cell survival during nutrient and oxygen deprivation. Our research reveals, for the first time, the participation of this receptor in steering cardiac fibroblasts towards a healing profile when subjected to stress-induced deprivation. Its role seems to be explained by its involvement in modulating autophagic flow, potentially disrupting the balance in eliminating pro-fibrotic factors within these cells.

Relationship Between Purinergic P2X Receptors and Pannexins in Retinal Dystrophies

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The purinergic receptors P2X7 (P2X7R) mediate neuroinflammation in retinal neurodegenerative diseases through glial activation, leading to the release of inflammatory cytokines and apoptosis. They also contribute to neuronal death eliciting intracellular Ca2+ overload, cytolysis and activation of inflammasome-dependent cell death pathway. P2X7R are widely co-expressed with P2X4R. Both receptors are mainly expressed in glial cells, ganglion cells and in synapsis sites. P2X7R can be activated and modulated by the release of ATP through Pannexin 1 (PanX1) channels. Considering that P2XRs and PanXs have been involved in several neurodegenerative disorders, we are interested in the study of the implication of P2XRs and PanX receptors in retinal dystrophies, and their contribution to the inflammatory state that is always present in these conditions. Hence, we have studied the expression of P2X7R, P2X4R, PanX1 and PanX2 in the retinas of two mouse models of inherited retinal dystrophies: Central areolar choroidal dystrophy (CACD) and retinitis pigmentosa (P23H). Our results reveal an upregulation of P2X7R and P2X4R in the retinas of P23H and CACD mice, compared with the expression in healthy (control) mice. They were preferentially overexpressed in immune cells (CD11b+ population). We have found a different pattern of P2XRs co-expression depending on the retinal dystrophy. We have also found differences in the upregulation of PanX1 and PanX2.These results suggest that the expression pattern of P2XRs and Panxs and their regulation differs among the dystrophies. However, more studies are needed to elucidate the mechanisms that are involved.

Targeting P2X receptors in ocular diseases, an example of retinal detachment and dry eye disease

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Receptors for nucleotides (UTP, ATP) formed by adding phosphate to nucleosides have been detected in many ocular tissues. These are called purinoceptors. There are two types of receptors. P2X and P2XY. While P2X is ionotropic, P2Y is metabotropic. Ionotropics are associated with channels in the cell membrane of small cations such as Na and Ca in cells where rapid transmission occurs, such as smooth muscle, autonomic nerves and neurons. Metabotropic receptors activate phospholipase C and G enzymes and partially adenylyl cyclase enzyme within the cell. The purinergic system plays a role in the physiology of the lacrimal apparatus, cornea, ciliary body, lens and retina. Nucleotides have become new treatment targets in dry eye patients by increasing tear secretion and mucin secretion from goblet cells. Additionally, nucleotides accelerate corneal reepithelialization via P2Y receptors. Stimulation of P2X2 receptors also increases the release of acetylcholine, relaxing the trabecular meshwork cells, thus increasing the aqueous outflow of the humor. P2X receptors in the retina are involved in the transfer of light stimulation from photoreceptor cells to ganglion cells. It has been determined that P2Y2 receptors in retinal pigment cells are important in determining the volume and composition of the subretinal space. Nucleotides such as ATP and UTP increase Cl permeation in the basolateral membrane of retinal pigment epithelial cells and reduce potassium permeation. Thanks to this regulation in ion transitions, the continuity of the tight connection between the retina and retinal pigment epithelial cells is ensured. Thus, the use of P2Y2 receptor agonists in the treatment of retinal detachment has come to the fore.

Discovery of new P2X7 Negative Allosteric Modulators for the Treatment of Inflammatory Eye Diseases

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High concentrations of extracellular ATP (eATP) are released by dying cells, for example during inflammation, causing the overactivation of the ATP-gated ion channel P2X7 which further promotes cell death and the release of pro-inflammatory cytokines.

The detection of high levels of ATP in the vitreous humour of patients affected by Age-related Macular Degeneration (AMD), an inflammatory eye disease causing progressive loss of central vision, and the identification of P2X7 receptors in the posterior eye, suggest that pro-inflammatory signalling via P2X7 receptors activation may be involved in the progression of AMD. Blocking P2X7 receptor activation may therefore be a valid therapeutic strategy to delay the progression of AMD.

In order to identify and develop new P2X7 antagonists, we used Computer Aided Drug Design (CADD). First, we used a ligand-based screening approach to identify five common chemical features among three known P2X7 antagonists (A740003, A804598 and JNJ47965567), screening a library of commercially available drug like compounds to select those with similar characteristics.

Second, we generated a human P2X7 homology model (based on the JNJ47965567-bound panda P2X7 crystal structure; PDB 5U1X) and virtually screened a library of compounds in this negative allosteric pocket (Schrödinger). The docked compounds were evaluated by consensus scoring using four algorithms (Glide XP, FlexX, PLANTS and FRED). After visual evaluation of the docking conformations, a total of 33 compounds were purchased to assess their activity in vitro.

A combination of two functional assays, YO-PRO 1 dye uptake and Membrane Potential Red (MPR), was used to identify two active compounds (7 and 26) which presented IC50 values in the low micromolar range. Further evaluation of structural analogues of these hits led to the discovery of an equally potent antagonist belonging to the compound 7 family (7g, IC50 1.31± 0.2 μM).

In order to develop assays for P2X7 receptor function in eye cells and tissues, we attempted to confirm expression of human P2X7 in both a human retinal pigment epithelial cell line (ARPE-19), and in two ex-vivo animal eye models (murine and porcine).

ARPE-19 cells were stimulated by ATP and BzATP in YO-PRO 1 dye uptake and MPR assays, but the small functional response observed at high (>1 mM) ATP concentrations was not diminished by JNJ47965567 pretreatment (0.1 μM and 20 μM). hP2X7 protein was not detected in this cell line by Western blotting, even following challenge with chemical stressors resembling hypoxia ($CoCl_2$) and cell damage (BzATP).

To confirm the presence of P2X7 in the whole eye, Western blotting was performed with tissue from murine whole eye, and porcine retina and choroid. P2X7 expression was detected in murine whole eye tissue extracts and in the porcine choroid (but not retina).

Our data demonstrate the identification of novel allosteric P2X7 antagonists using the CADD approach, and that P2X7 receptors are expressed in whole murine eye tissue and in porcine choroid, but show no evidence for P2X7 receptor expression in the ARPE-19 human cell line.

Efficacy of inflammasome inhibition to prevent myocardial ischemia-reperfusion injuries: a protocol for a systematic review and meta-analysis of animal studies

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Introduction: Following prolonged ischemia such as in myocardial infarction, reperfusion is accompanied by additive lesions known as myocardial ischemia-reperfusion injuries (mIRI). One of the pathophysiological substrates of mIRI involves sterile inflammation with secondary activation of the inflammasome pathway and mitochondrial dysfunctions. Over the past 20 years, a myriad of pharmacological interventions targeting this pathway, and in particular its NLRP3 axis (NOD-like receptor protein 3) / pro-caspase-1 / interleukin-18 / interleukin-1β (e.g., colchicine, anakinra, canakinumab), has been studied in various experimental models. Despite an extensive literature, attempts to translate these findings into clinical practice has produced contradictory results. Factors explaining these results are numerous and depend not only on the intrinsic performance of the animal models used, but also on the characteristics of the pharmacological interventions (type of drugs, dosage, time and duration of administration) and those of the target population (stage and severity, comorbidities). The aim of our study is to synthesize and quantify preclinical evidence regarding the modulation of inflammasome to reduce mIRI in animal studies to identify the most promising targets that could be translated to clinic.

Methods: We will perform a systematic review and meta-analysis following PRISMA guidelines. Pubmed/Medline, Embase and Web of Science will be screened to systematically review preclinical evidence assessing the efficacy of inflammasome modulation in controlled preclinical in vivo models of acute mIRI. Literature search algorithm, as sensitive as possible, will cover four key dimensions: 'myocardium/myocardial', 'reperfusion injury', 'animal studies', and 'drugs and pharmacological target'. Methodological quality of animal studies will be assessed using a tool derived from the SYRCLE and CAMARADES Risk of Bias. The primary outcome will be the infarct size (per area at risk, per total ventricular area). The secondary outcomes will include left ventricular parameters (functional and anatomical) and biomarkers (troponin, creatine kinase, cytokines, growth factor, extracellular matrix, NLRP3 inflammasome components). For each specified outcome, effect sizes will be pooled using a random-effects meta-analytical approach to derive the standardized mean difference, accompanied by a 95% confidence interval.

Conclusion: The results of this systematic review and meta-analysis will provide translational scientists and clinical trialists with the current preclinical evidence related to the efficacy of inflammasome modulation on mIRI animal models. Il will also help identify evidence gaps, explore response factors and source of variability and guide future related research.

Systematic review registration: The protocol will be submitted to PROSPERO for registration and will be submitted to Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) for public posting.

Retinal ganglion cell death in a model of sepsis and its rescue by P2X7R antagonism

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Sepsis induces neurological dysfunction, although it is unclear whether this is due to neurodegeneration or just glial activation and neuroinflammation. There is a sexual dimorphism in the response to systemic inflammation, but comprehensive studies are lacking. In a model of sepsis induced by LPS administration, we have shown that both male and female mice exhibit transient retinal dysfunction, loss of vision forming but not non-vision forming RGCs, and increased levels of TNF and IL-1β in their retinas. Males had greater loss of vision-forming RGCs, delayed functional recovery and increased lymphotoxin alpha expression compared to females. Antagonism of P2X7R and TNFR1, either individually or in combination, showed significant rescue of RGCs, with P2X7R antagonism additionally restoring retinal function. Female mice showed a better response to treatment than males. Thus, systemic LPS-induced inflammation exerts neuronal and sex-specific effects on the mouse retina that are attenuated by targeting the P2X7R and the extrinsic pathway of apoptosis. These findings highlight the need to include sex-specific analyses in preclinical studies of inflammatory conditions affecting the central nervous system.

P2X7 expressed by macrophages controls T-cell mediated autoimmune response

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During disease, damaged cells release high levels of extracellular ATP, which constitutes a potent danger signal. The purinergic receptor P2X7 is the main sensor of high amounts of extracellular ATP and its activation triggers an inflammatory response and/or induces cell death. P2X7 expression is increased in various neurodegenerative and autoimmune diseases, in particular P2X7 was shown to be up-regulated in monocytes from patients with autoimmune Behcet's uveitis. Uveitis are inflammatory eye diseases of various origins which can be debilitating and lead to blindness. Thus, P2X7 may contribute to the development of uveitis via its expression on immune cells. Our data showed that P2X7 is expressed in the different immune cell subpopulations in the retina during experimental autoimmune uveitis (EAU). Given the multiple functions of P2X7, its activation could play distinct roles in the development of the disease depending on the cell type that expresses it. We investigated the function of P2X7 using active and passive EAU models and new conditional P2X7 knockout mouse strains. Although we found no significant effect of P2X7 invalidation in T-cells, we showed that the severity of EAU was significantly reduced when P2X7 was specifically depleted in retinal microglia and infiltrating macrophages. The decreased severity was associated with reduced expression of inflammatory mediators. Thus, targeted inhibition of P2X7 could be a promising therapeutic approach for the treatment of autoimmune diseases.

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