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THE SYSTEMIC INFLAMMATORY RESPONSE IN HODGKIN LYMPHOMA SURVIVORS: ARE ADVANCED GLYCATION END PRODUCTS POTENTIAL PLAYERS?

(1) E. Aimaretti, (2) F. Felicetti, (3) F. Dal Bello, (2) F. Gatti, (4) M. Cassader, (5) M. C. Cifani, (6) F. Fagioli, (1) M. Aragno, (7) M. Collino, (2) E. Brignardello

(1) Department of Clinical and Biological Sciences, University of Turin, Turin, Italy
(2) Transition Unit for Childhood Cancer Survivors, Città della Salute e della Scienza Hospital, Turin, Italy
(3) Department of Molecular Biotechnology and Health Science, University of Turin, Turin, Italy
(4) Department of Medical Science, University of Turin, Turin, Italy
(5) School of Pharmacy, University of Camerino, Camerino, Italy
(6) Division of Paediatric Onco-Haematology, Stem Cell Transplantation and Cellular Therapy, Città della Salute e della Scienza Hospital, Turin, Italy
(7) Rita Levi Montalcini Department of Neurosciences, University of Turin, Turin, Italy

INTRODUCTION: current pediatric treatment protocols for Hodgkin lymphoma (HL) include a combination of risk-adapted chemotherapy together with low-dose involved-field radiotherapy. These therapeutic approaches can trigger an inflammatory response by causing necrosis and tissue injury that stimulate late complications. Hodgkin lymphoma survivors show an increased risk of long-term complications, particularly cardiovascular diseases and malignant neoplasms.
Advanced Glycation End products (AGEs) are formed under hyperglycemic conditions, but also as a consequence of inflammation and unbalanced oxidative stress, which have been both suggested as a potential determinant of cardiovascular disorders and cancer.
METHODS: 20 HL survivors (HLS) and 40 age and sex-matched healthy controls (C) were enrolled in the study. After the isolation of peripheral blood mononuclear cells (PBMC) and the collection of plasma, we performed analyses of gene expression by qRT-PCR and the analysis of inflammatory and oxidative-stress markers.
RESULTS: HL survivors showed a condition of higher oxidative stress, as demonstrated by an increased expression of NADPH oxidase on PBMC. Antioxidant defenses, evaluated in terms of alpha-tocopherol, GSSG/GSH ratio and catalase plasma levels, were strongly reduced in survivors. This pro-oxidative condition led to the over-expression of both NLRP3 and NFkB genes in PBMC and, consequently, to an increased plasma levels of interleukin (IL)-1β and IL-6. Moreover, AGEs plasma levels, expressed as Nε-carboxymethyl-lysine (CML) and methylglyoxal hydroimidazolone (MG-H1), were markedly higher in HL survivors than in healthy subjects. The expression of the receptors for AGEs in PBMC confirmed the dysregulated AGE pathways.
CONCLUSIONS: in pediatric HL survivors, the accumulation of AGEs and the subsequent activation of their receptor RAGE is associated to the activation of proinflammatory intracellular signalling cascades leading to a chronic low grade inflammatory response, that persists after the end of anticancer treatments and that may contribute to the onset of late complications. Overall, these data suggest AGEs as potential new pharmacological target for counteracting late complications in HL survivors.

IMMUNOMODULATORY EFFECTS OF ATRAZINE (ATR) ON HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

M. N. Alhosseini, L. Cari, G. Migliorati, G. Nocentini

Section of Pharmacology, Department of Medicine and Surgery, University of Perugia, Perugia, Italy

BACKGROUND: for many years, the possible health threat posed by endocrine disruptors (EDs) which are substances in our environment, food, and consumer products has drawn the attention of researchers. EDs are well known for causing an alteration of the homeostasis of sexual hormones. However, EDs interfere also with the immune system through the apoptosis induction of leukocytes and causing alteration of NK cell, DC, and macrophage functions. Only a few studies evaluated the ED effects on the differentiation of T cells resulting in the expansion of Th1, Th2, Th17, or regulatory T cells (Tregs) subsets. The aim of the present study is to investigate the immunomodulatory effect of the ED atrazine (ATR), a widely used pesticide, on human Peripheral Blood Mononuclear Cells (PBMC).
METHODS: fresh PBMCs were isolated from human buffy coats obtained from healthy and young donors, using Lympholyte. The cells were seeded in 12-well plates in 2 ml of medium at a concentration of 1 x 10^6 cells/ml, activated by human T-Activator CD3/CD28 beads and treated with different concentrations of atrazine including 0.1, 1, 10, and 100 µM. Medium-treated and atrazine solvent (DMSO)-treated controls were also performed. Each treatment and control were performed in duplicate and quadruplicate, respectively. Following four days of incubation, harvesting, permeabilization and staining, the expression of cytokines and T cell markers including CD4, CD8, CD25, GITR and FoxP3 was evaluated by flow cytometry.
RESULTS: in each of the five tested donors, atrazine exposure significantly decreased the number of $CD4^+$, $CD8^+$, and $CD4^+CD8^+$ T cells producing IFN-γ. At the highest atrazine concentrations (10 and 100 µM) the reduction of IFN-γ^+ cells is equal or more than 50%. Of note, the effect was observed in a concentration-dependent manner. The decrease of IFN-γ^+ cells was also relative to all the cells that express cytokines (relative increase). A concentration-dependent relative increase of IL-4^+ $CD4^+$ cells is also observed in several PBMC donors.
The percentage of $CD4^+$ T cells expressing at intermediate/high-level FoxP3, the main marker of thymus-derived Treg (tTreg) and some peripherally-derived Treg (pTreg), showed a 20-30% increase at the highest atrazine concentrations (10 and 100 µM), in several but not all donors' PBMC. At the same time, the percentage of $GITR^{high}CD25$-T cells, representing a relevant subset of pTreg, is decreased by atrazine treatment.
CONCLUSIONS: these results suggest the immunomodulatory effects of atrazine are several and complex. The decrease of IFN-γ^+ $CD4^+$ cells and the increase of IL-4^+ $CD4^+$ cells suggest that atrazine favors a Th2 polarization. About Treg, atrazine seems to increase $FoxP3^+$ cells but to decrease a subset of pTreg (the GITRsp subsets) with overall effects that are difficult to be predicted. Further work is required to clarify the shift in immune subsets and alterations in the ability of exposed individuals to arise protective adaptive immune responses.

BENEFICIAL EFFECTS OF ULVA PERTUSA IN AN EXPERIMENTAL MODEL OF INFLAMMATORY BOWEL DISEASES

A. Ardizzone, L. Cucinotta, M. Campolo, V. Bova, S. Cuzzocrea, E. Esposito

Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy

BACKGROUND: inflammatory bowel diseases (IBD) constitute multifactorial chronic gastrointestinal disorders characterized by relapse-remitting symptomatology. The etiopathogenesis of IBD is not yet fully understood, however, it has been demonstrated that innate immune dysfunction and inflammatory condition contributes to the development of these disorders. Natural compounds have proved to be a promising alternative in the management of various human diseases, including IBD, showing considerable pharmacological activities and fewer side effects compared to conventional therapy. In particular, *Ulva pertusa*, a green alga of the *Ulvaceae* family, has been shown to possess important biological properties which are still in continuous deepening. In this perspective, this study aimed to explore the immunomodulatory and anti-inflammatory capabilities of *Ulva pertusa* in a murine model of DNBS-induced colitis.
METHODS: experimental colitis was induced by intrarectal instillation of dinitrobenzene sulfonic acid (DNBS; 4 mg in 100 µl of 50% ethanol). *Ulva pertusa* (10, 50 and 100 mg/kg) was administered daily by oral gavage. At the end of the experiment, 4 days after injury induction, mice were sacrificed, colon removed by surgical procedure and processed for biochemical analysis and histological examinations.
RESULTS: the obtained data showed that *Ulva pertusa* treatment significantly reduced weight loss, tissue injury and neutrophil infiltration as well as mast cells degranulation. In addition, *Ulva pertusa* administration decreased the expression of pro-inflammatory enzymes, modulating NF-kB pathway and Th cytokine-dependent levels such as IL-4, IL-5, IL-9 and IL-13.
CONCLUSIONS: these results showed that *Ulva pertusa* acts as immunomodulator for the inflammatory response in IBD. Therefore, *Ulva pertusa* extract can be considered a valuable strategy to reduce the development of IBD, improving patients' quality of life.

GENDER-SPECIFIC MODULATION OF ENDOTHELIAL PD-L1 TRAFFICKING

(1) C. Baggio, (2) F. Oliviero, (2) P. Sfriso, (1) C. Bolego, (2) A. Cignarella

(1) Department of Pharmaceutical and Pharmacological Sciences, University of Padua, Padua, Italy
(2) Department of Medicine, University of Padua, Padua, Italy

BACKGROUND: immune checkpoints are negative regulators of the immune response. The crosstalk between programmed cell death-1 protein (PD-1) and its ligand (PD-L1) is relevant in cancer and in autoimmune diseases including rheumatoid arthritis (RA), which is more prevalent in women than in men. Pharmacological blockade of PD-1 or PD-L1 often leads to immune-related adverse events. PD-L1 is constitutively expressed in various cell types including endothelial cells (ECs) and can be upregulated by inflammatory stimuli as a suppressive signal. Of note, VEGF has been reported to upregulate the expression of PD-L1/PD1, and anti-VEGF agents show immunosupportive properties. Soluble forms of PD-1 (sPD-1) and PD-L1 (sPD-L1) have been detected in RA patients' serum or plasma. However, it is unclear (a) whether and to what extent endothelial PD-L1 is a source of circulating sPD-L1 via proteolytic cleavage; and (b) if gender differences occur in the regulation of this system.
METHODS: HUVECs were isolated from human umbilical cords by female (fHUVECs) and male (mHUVECs) newborns. The surface and intracellular expression of PD-L1 were measured by flow cytometry and western blot, respectively. HUVECs were stimulated with IFN-γ (1000 IU/mL), IL-1β (2 ng/mL), IL-6 (50 ng/mL) or VEGF (50 ng/mL) for 3 to 24 hours. Selected experiments were carried out in the presence of bevacizumab (1 mg/mL) or the pan-metalloprotease (MPP) inhibitor GM6001 (10 μM). Quantitative analysis of sPD-L1 in HUVEC supernatants at different times (3 to 24 h) was performed by ELISA. Synovial fluids were obtained from patients with RA (n = 6) and osteoarthritis (OA, n = 3). Levels of inflammatory cytokines and VEGF levels were measured by specific ELISA assays.
RESULTS: membrane PD-L1 was expressed on resting HUVECs, and no baseline differences between fHUVECs and mHUVECs were observed. Stimulation with IFN-γ or IL-1β for 24 h failed to increase membrane PD-L1 abundance. Conversely, the amount of intracellular PD-L1 after 24-h stimulation with IFN-γ, IL-1β, IL-6 or VEGF increased in fHUVECs but not in mHUVECs. In addition, sPD-L1 released by IL-1β-stimulated fHUVECs increased in a time-dependent manner. Similarly, sPD-L1 was also increased upon treatment of fHUVECs with VEGF for 6 or 24 h, an effect that was significantly inhibited by bevacizumab. In fHUVEC pretreated with the MPP inhibitor GM6001, IL-1β stimulation did not increase sPDL-1 release, suggesting that proteolytic cleavage of membrane PD-L1 occurred over time. In mHUVECs sPD-L1 levels were unchanged in response to any stimuli. Finally, synovial fluids from RA contained higher levels of inflammatory cytokines and VEGF compared with those from OA patients, and increased PD-L1 intracellular accumulation in fHUVECs but not in mHUVECs.
CONCLUSIONS: endothelial PD-L1 was expressed and released by ECs in response to various inflammatory stimuli in a gender-specific manner, suggesting a feedback compensatory mechanism to control autoimmunity in females. Endothelial sPD-L1 was released by proteolytic cleavage in response to cytokines and growth factors. This process may prevent immune cell activation and endothelial dysfunction associated with chronic inflammatory disease.

IDENTIFICATION OF NEW COMPOUND CHIN117 WITH DUAL ACTIVITY, GPBAR1 AGONIST AND CYSLT1R ANTAGONIST, FOR THE TREATMENT OF ACUTE AND CHRONIC HEPATITIS

(1) M. Biagioli, (1) S. Marchianò, (1) C. Di Giorgio, (2) R. Roselli, (1) R. Bellini, (1) M. Bordoni, (1) A. Carino, (2) A. Zampella, (1) S. Fiorucci

(1) Department of Medicine and Surgery, University of Perugia, Perugia, Italy
(2) University of Naples Federico II, Department of Pharmacy, Naples, Italy

BACKGROUND: Cystenyl-leukotrienes (CysLTs) are pro-inflammatory lipid molecules, derived from arachidonic acid (AA) metabolism. CysLTs family includes LTC4, LTD4 and LTE4 (1). Under to a specific stimulus white blood cells, especially the cells of innate immunity, secrete CysLTs that bind and activate specific G protein-coupled receptors (GPCRs) perpetuating a powerful inflammatory response (2). CysLT1R is the best characterized receptor and represents an interesting target for the treatment of inflammation therefore many selective antagonists are commercially available as Montelukast, Zafirlukast and Pranlukast (3). Our analysis showed that the CysLT1R-antagonists might well reproduce the binding mode of some known agonists of a bile acid receptor known as GPBAR1 (also known as Takeda G protein Receptor, TGR5) (4-6). In accordance with these data, Biagioli *et al.* in 2020 showed that another CysLT1R antagonist, the alpha-pentyl-3-[2-quinolinylmethoxy] benzyl alcohol (REV5901), attenuates inflammation and immune dysfunction in vitro and in mouse model of colitis thanks also to the action on GPBAR1, while the effect is lost in Gpbar1 knock mice confirming that REV5901 is a GPBAR1 ligand (7). GPBAR1 is a G-protein-coupled cell transmembrane receptor, activated by a wide range of ligands such as endogenous secondary bile acids (LCA and DCA) and their taurine conjugates (TLCA and TDCA) (8). Gpbar1 expression is found on monocyte, macrophage NK and NKT cells and its activation induces a down-regulation of inflammatory (9-12). Starting from these assumptions, we are focusing on developing a new CysLT1R antagonists able to interact with bile acid activated receptors GPBAR1 using REV5901 as a template for its dual activity (7).
METHODS: therefore, with the aim at reproducing the molecular properties of montelukast and using REV5901 as starting structure, we have synthesized a series of biphenyloxy-methyl-quinoline compounds endowed with either a hydroxyl, a carboxylic or an ester group purposely spaced apart from the quinoline ring. The derivatives were tested for FXR and GPBAR1 activity, in a luciferase reporter assay with HepG2 and HEK-293T cells transfected with FXR and GPBAR1, respectively. Evaluation of the antagonist activity of compounds at the human CysLT1 receptor expressed in transfected CHO cells was determined by measuring their effect on agonist-induced cytosolic $Ca2^{+}$ ion mobilization using a fluorimetric detection method. The most effective and potent compound was then tested in mouse models of acute (APAP-induced hepatitis) and chronic (High Fat Diet with fructose, HFD-F) hepatitis in comparison to Montelukast, a reference antagonist for Cyslt1R, and BAR501, selective agonist of GPBAR1, also using Gpbar1$^{-/-}$ mice.
RESULTS: CHIN117 relieved acute APAP-induced hepatitis in a dose-dependent manner showing greater efficacy of both BAR501 and Montelukast at all doses. RNAseq analysis of the livers of mice treated with the various compounds showed that the effect on the modulation of gene expression of CHIN117 overlaps more with the effect exerted by Montelukast (overlap about 3000 genes) than with BAR501 (overlap about 800 genes). To investigate the role of GPBAR1 in the mechanism of action of CHIN117 we induced hepatitis by administering APAP in Gpbar1$^{+/+}$ and Gpbar1$^{-/-}$ mice. Biochemical and histological data, and analysis of inflammatory status by qPCR and IC-FACS showed that Gpbar1$^{-/-}$ mice developed more severe disease than wild-type. In knock-out mice the beneficial effect of CHIN117 treatment is only partially maintained confirming that GPBAR1 is a target of the compound. Afterwards we investigated the efficacy of the new compound CHIN117 also in a mouse model of high-fat diet-induced chronic hepatitis which simulates NAFLD. The weight trend showed that CHIN117 reduced the weight gain by approximately 3 grams, reversed insulin resistance and reduced significantly AST, ALT, and LDL levels counteracting the hepatotoxic effect exerted by the HFD-F diet. Moreover, CHIN117 almost completely reversed the disease by reducing liver steatosis, BMI and the weight of eWAT, BAT and liver.
CONCLUSIONS: we have shown that a novel dual-activity compound, GPBAR1 agonist and CYSLT1R antagonist, may have utility in the treatment of acute and chronic liver disease.

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OXER1 AND RACK1-ASSOCIATED PATHWAY: A PROMISING DRUG TARGET IN TRIPLE NEGATIVE BREAST CANCER AND ITS EMERGING ROLE IN THE TUMOR MICROENVIRONMENT (TME)

(1) E. Buoso, (1, 2) M. Masi, (3) E. Garattini, (3, 4, 5) M. Bolis, (6) D. Di Marino, (1) L. Maraccani, (1) E. Morelli, (7) A. A. Grolla, (1, 2) F. Fagiani, (8) E. Corsini, (1) C. Travelli, (1) S. Govoni, (1) M. Racchi

(1) Laboratory of Pharmacology, Department of Drug Sciences, University of Pavia, Pavia, Italy
(2) University School for Advanced Studies IUSS, Pavia, Italy
(3) Laboratory of Molecular Biology, Istituto di Ricerche Farmacologiche Mario Negri IRCCS, Milan, Italy
(4) Functional Cancer Genomics Laboratory, Institute of Oncology Research, USI, University of Southern Switzerland, Bellinzona, Switzerland
(5) Bioinformatics Core Unit Institute of Oncology Research, Swiss Institute of Bioinformatics, Lausanne, Switzerland
(6) Department of Life and Environmental Sciences, New York-Marche Structural Biology Center (NYMaSBiC), Polytechnic University of Marche, Ancona, Italy
(7) Department of Pharmaceutical Sciences, University of Piemonte Orientale, Novara, Italy
(8) Laboratory of Toxicology, Department of Environmental Sciences and Policy, University of Milan, Milan, Italy

BACKGROUND: in Breast Cancer (BC), the expression of RACK1 – a putative prognostic marker involved in cancer cell proliferation, migration, and invasion – is negatively correlated with overall survival. Since a complex glucocorticoids-androgens balance controls RACK1 expression (thanks to a Glucocorticoid Responsive Element in its promoter), we assessed the role of androgen signaling in RACK1 transcriptional regulation in BC. In this regard, we demonstrated that membrane androgen receptor OXER1 activates PI3K/Akt/NF-κB/RACK1 and FAK signaling pathways to promote survival, cell adhesion and migration and is now emerging as an important player in triple negative BC (TNBC). Our *in silico* GSEA on TNBC patients' samples identified "Interferon alpha (IFNα) response" as top-enriched upregulated gene set. Indeed, under conditions of chronic IFNα signaling, IFNα can have immunosuppressive roles that promote pro-tumorigenicity and contribute to metastasis formation and BC progression. These features can be specifically attributed to the triple negative inflammatory BC (TN-IBC) subtype, which features higher infiltration of tumor-associated macrophages (TAMs) and tumor-suppressing M2 compared to TNBC. Therefore, the aim of our study is to elucidate OXER1 role in promoting TNBC progression and sustaining non-canonical interferon stimulated genes (ISGs) and tumor microenvironment-produced cytokines to promote TN-IBC aggressive phenotype.
METHODS: to assess whether RACK1 transcriptional regulation was AR- or mAR-dependent, involving PI3K/Akt/NF-κB pathway, luciferase reporter assay, qPCR, immunoblotting, cell proliferation (MTT), colony formation assay, cytofluorometry and scratch-wound healing assay were performed on MCF7 and MDA-MB-231 cells treated with testosterone, testosterone-BSA-FITC, or nandrolone. Immunofluorescence and *in silico* molecular docking confirmed that nandrolone exerts its effects via OXER1. We validated our panel with patient-based transcriptomic data. Analogue experiments and 3D spheroid models were performed on TN-IBC HCC1187 and CAL-85-1 cells, while specific experiments and sandwich ELISA assays were performed to evaluate macrophages migration and polarization.
RESULTS: Our data confirmed that RACK1 is involved in BC progression and we provided first evidence that nandrolone mediates negative effects on BC cell proliferation and migration due to its antagonization of PI3K/Akt/NF-κB pathway, ultimately leading to RACK1 down-regulation. Nandrolone impairs this signaling pathway through its binding to OXER1, whose increased expression is higher in tumors tissue compared to non-cancerous ones and correlated with ER and PR status in patients. The same effects on PI3K/Akt/NF-κB pathway were observed in HCC1187 and CAL-85-1 cells in terms of cell proliferation, migration and 3D spheroid formation, confirming OXER1 role as previously hypothesized. Finally, these data were in line with the reduced production of OXER1-correlated cytokines, in particular IFNα and IL-8 thus suggesting an emerging role in TME.
CONCLUSIONS: our data support the idea that androgen derivatives or repurposed drugs tailored to antagonize OXER1 activation pathway may represent promising and rational agents for the personalized treatment of BC.
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UTILIZATION PATTERNS AND HEALTHCARE ACCESSES OF JAKI USED IN RHEUMATOID ARTHRITIS PATIENTS IN TUSCANY: THE LEONARDO STUDY

(1) M. Tuccori, (2) V. Lorenzoni, (3) R. Gini, (2) G. Turchetti, (4) E. Fini, (5) S. Giometto, (6) C. Bartolini, (6) O. Paoletti, (7) I. Convertino, (7) S. Ferraro, (7) E. Cappello, (7) G. Valdiserra, (1) C. Blandizzi, (8) E. Lucenteforte

(1) Unit of Pharmacology and Pharmacovigilance, Department of Clinical and Experimental Medicine, University of Pisa; Unit of Adverse Drug Reactions Monitoring, University Hospital of Pisa, Pisa, Italy
(2) Institute of Management, Scuola Superiore Sant'Anna, Pisa, Italy
(3) Agenzia regionale di sanità della Toscana, Osservatorio di epidemiologia, Florence, Italy
(4) Medical Specialization School of Pharmacology, University of Pisa, Pisa, Italy
(5) Unit of Medical Statistics, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
(6) Agenzia regionale di sanità della Toscana, Florence, Italy
(7) Unit of Pharmacology and Pharmacovigilance, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
(8) Unit of Medical Statistics, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy

BACKGROUND: the first JAK inhibitor (JAKi) was approved for rheumatoid arthritis (RA) in late 2018. This study is aimed at characterizing JAKi users by evaluating their prescription history and the healthcare utilization patterns using administrative healthcare databases.
METHODS: this is a descriptive, retrospective cohort study on Tuscan administrative healthcare databases. New Tuscan users of JaKi were divided into two groups according to the date of the first dispensation: 1) from January 1st, 2018 to December 31st, 2019 (cohort entry 1), 2) from January 1st, 2018 to June 30th, 2019 (cohort entry 2). Adult patients with at least 10 years of baseline period and at least six months of follow-up were included. Distribution of first JAKi users, DMARD dispensations in the lookback period, mean time, and standard deviation (SD) from first DMARD to JAKi were calculated in the first analysis. Number of emergency department (ED) admissions and hospitalizations (H) for any cause and number of RA visits (RV) in the follow-up, mean time and standard deviation (SD) from the first event of interest were estimated in the second analysis.
RESULTS: in the 363 new JAKi users included in the first analysis, the mean age was 61.5 years (SD 13.6) and 80.7% were women. The 79% of patients had conventional synthetic DMARD before the cohort entry, the most commonly used of which were hydroxychloroquine (44%), methotrexate (42%), and leflunomide (33%). The 60% had a history of biologic DMARD with a high distribution of etanercept (40%), abatacept (30%) and adalimumab (27%). As a second (31.1%), third (33.1%), or fourth line (27.8%) treatment, JAKi were administered in a similar manner. The mean time for JAKi was 7.2 years (SD 3.3). 221 new JAKi users were included in the second analysis. 109 accesses to ED, 39 H and 64 RV were observed. All RVs were recorded in patients treated with baricitinib. The mean time in days to first hospitalization in ED was 73.5 (SD 54.1), at first H it was 89.6 (SD 54.8) and at first RV it was 60.9 (SD 51.3). The causes of access to the emergency room most frequently were classified as injuries and intoxications (18.3%), skin (13.8%) and circulatory disorders (11.0%), while those associated with hospitalizations were cardiovascular (69,2%), musculoskeletal (64.1%) and skin manifestations (38.5%).
CONCLUSIONS: our findings showed that JAKi are used in accordance with the current RA clinical guidelines. Between the two drugs, the utilization of healthcare services was similar and the events reported were in line with the known safety profile or the underline disease.

BIOINFORMATICS ANALYSIS OF THE IMMUNE INFILTRATE IN HODGKIN'S LYMPHOMA: PROGNOSTIC AND THERAPEUTIC IMPLICATIONS

L. Cari, M. N. Alhosseini, C. Colopi, G. Migliorati, G.Nocentini

Section of Pharmacology, Department of Medicine and Surgery, University of Perugia, Perugia, Italy

BACKGROUND: Hodgkin's lymphoma is a cancer of the lymphatic system that affects about 4 out of 100,000 people. In this cancer, tumor cells (Reed Sternberg cells) represent only a small part (2-3%) of the neoplastic mass and are surrounded by an immune cell infiltrate. Although the currently used therapies are effective, these have various adverse effects that significantly affect patients' quality of life. Furthermore, some patients relapse, and others are refractory to therapy.
This work aimed to evaluate the immune infiltrate of Hodgkin's lymphoma and how it differs in disease stages and patients of different ages 1) to find prognostic markers for the response to therapy and 2) to develop new therapeutic approaches.
METHODS: using the GeneVestigator software, array data stored in the databases were analyzed; in particular, 125 lymph node samples from patients with Hodgkin's lymphoma 19 lymph node samples from healthy subjects, as a control group, were analyzed. Through the use of signatures developed in our laboratory, we assessed the infiltration levels of B and T lymphocytes, macrophages, $CD8^+$ and Natural Killer ($CD8^+$/NK) cells. Furthermore, the expression levels of regulatory T cells (Treg) markers and immunosuppressive and proinflammatory cytokines were evaluated.
RESULTS: the infiltration levels of T and $CD8^+$/NK cells and macrophages increase significantly in the lymph nodes of patients compared to those of healthy controls. Interestingly, interleukin (IL)-10 mRNA levels are considerably higher in diseased than in healthy tissue and closely correlate with the presence of macrophages; in addition, macrophages levels increase with age increase of the patients and the progress of the disease. Moreover, we observed that a high level of infiltration by macrophages and IL-10 represents a negative prognostic index; on the contrary, a high level of infiltration of B lymphocytes and IL-17 mRNA are positive prognostic indices.
Therefore, we propose an algorithm (Hodgkin's lymphoma prognostic algorithm - HLPA) that, using these parameters, predicts the response to therapy; starting with a patients population showing 70% complete response, 20% relapse, and 10% lack of response to treatment, with an HLPA < 1.69 the complete response rate is equal to 95% (classifying 17% of the patients) and with an HLPA < 3.59 the complete response rate is equal to 84% (classifying 45% of the patients).
CONCLUSIONS: HLPA is a tool potentially able to predict if a patient responds to treatment and may help the physician in the decision-making process. The parameters of the HLPS are currently being fine-tuned to be able to classify a greater number of patients.
Our data link the high plasma levels of IL-10 found in Hodgkin's lymphoma patients with the production of this at the level of the tumoral lymph nodes, suggest that IL-10 is crucial in the development and maintenance of the disease and that monoclonal antibodies against this cytokine may be of help in the response of treatment.

LONG-CHAIN VITAMIN E METABOLITES SUPPRESS AIRWAY HYPERREACTIVITY IN EXPERIMENTAL ASTHMA

(1) I.Cerqua, (2) K. Neukirch, (3) M. Terlizzi, (1) E. Granato, (1) E. Caiazzo, (1) G. Cirino, (1) A. Ialenti, (4) J.J. Helesbeux, (3) R. Sorrentino, (2) A. Koeberle, (1) E. F. Roviezzo and (1) A. Rossi.

(1) Department of Pharmacy, School of Medicine, Federico II University of Naples, Naples, Italy
(2) Department pf Pharmaceutical/Medicine, Michael Popp Institute and Center for Molecular Biosciences Innsbruck (CMBI), University of Innsbruck, Innsbruck, Austria
(3) Department of Pharmacy (DIFARMA), University of Salerno, Fisciano, Salerno, Italy
(4) University of Angers, SONAS, SFR QUASAV, Angers, France

BACKGROUND: vitamin E is a potent lipid antioxidant that includes both tocopherols and tocotrienols present in different types of foods (nuts, corn, oil, *etc.*) (1). Recently, it has been demonstrated that vitamin E mediates immune functions through endogenous long-chain metabolites (LCMs) by targeting 5-lipoxygenase (5-LOX) and increasing the systemic concentrations of Resolving E3, a specialized pro-resolving lipid mediator (2). Of note, vitamin E deficiency affects the immune system inducing the development of different diseases (3). Particularly, low plasma levels of α-tocopherol are associated with an increase in asthma incidence in adults or children (4). This study aims to investigate the role of LCMs in a model of allergic lung inflammation.
METHODS: adult BALB/c female mice were subcutaneously treated with ovalbumin (OVA 100 μg) at the day 0 and 8. Part of the mice was intraperitoneally (i.p.) treated with the endogenous vitamin E metabolite 13'-carboxy-α-tocopherol (α-T-13'-COOH; 10 mg/kg i.p. 30 min before OVA) or the semi-synthetic lead compound α-amplexichromanol (α-AC; 10 mg/kg i.p. 30 min before OVA). Then, all the mice were sacrificed following 21 days of treatment to perform functional and molecular studies.
RESULTS: the i.p. pretreatment with α-T-13'-COOH or α-AC significantly reduced the OVA-induced bronchial hyperreactivity, and this was coupled to a reduction in IgE plasma level and pulmonary levels of LTC_4, T $CD4^+$ cells and mast cells. In particular, the immunomodulatory effect of α-AC was confirmed by mass spectrometry analysis which proved an increase of pro-resolving mediators by targeting cyclooxygenase and 12/15-LOX in the lungs. Further, the immunohistochemical studies confirmed the protective role of these compounds with a reduction of pro-inflammatory cell infiltration in peri-bronchial sections.
CONCLUSIONS: this study provided strong evidence of the immunomodulatory role of α-T-13'-COOH and α-AC which inhibited the 5-LOX activity and increased the pro-resolving lipid mediators. This immunomodulatory effect of α-tocopherol supplementation could be considered as a new pharmacological approach in allergic airway diseases such as asthma.

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POTENTIAL REPOSITIONING OF BARICITINIB FOR PREVENTING DIET-INDUCED METABOLIC ABNORMALITIES: AN *IN-VIVO* STUDY

(1) D. Collotta, (2) W. Hull, R. (3) Mastrocola, (3) E. Aimaretti, (1) F. Chiazza, (3) A. S. Cento, (2) C. Murphy, (1) R. Verta, (1) G. Ferreira Alves, (4) G. Gaudioso, (4) F. Fava, (3) M. Aragno, (4) K. Tuohy, (2) C. Thiemermann, (5) M. Collino

(1) Department of Drug Science and Technology, University of Turin, Turin, Italy
(2) Queen Mary University of London, Centre for Translational Medicine and Therapeutics, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, London, United Kingdom
(3) Department of Clinical and Biological Sciences, University of Turin, Turin, Italy
(4) Edmund Mach Foundation, San Michele all'Adige, Italy
(5) Rita Levi Montalcini Department of Neurosciences, University of Turin, Turin, Italy

BACKGROUND: Janus kinase/signal transducer and activator of transcription (JAK-STAT) pathway exerts a pathologic effect exacerbating metaflammation, a chronic inflammatory state that characterizes diet-related metabolic disorders. We investigated the effects of the Jak1/2 inhibitor baricitinib, recently approved for the treatment of rheumatoid arthritis, in a murine model of high-fat-high-sugar diet (HD).
METHODS: 4-week old male C57BL/6 mice were fed with a control normal diet (ND) or a high-fat-high sugar diet (HD) for 22 weeks. A sub-group of HD fed mice was treated with baricitinib (10 mg/kg die, p.o.) for the last 16 weeks (HD + Bar).
RESULTS: HD group showed increased of: body weight, triglycerides, cholesterol, LDL, blood glucose levels ($P < 0.05$) and an impairment in OGTT, compared to ND. HD resulted in increased leptin, resisting and insulin plasma levels ($P < 0.05$), impaired insulin signaling transduction and in reduced GIP, GLP-1 and ghrelin ($P < 0.05$). HD also led to increased systemic proinflammatory markers IL-1β, INF-y; TNF-α ($P < 0.05$), and to reduced anti-inflammatory IL-10 and IL-6 ($P < 0.05$). Despite HD + Bar did not change diet-induced microbiota imbalances, the metabolic abnormalities were reverted.
CONCLUSIONS: in summary, our data suggest that Jak2/Stat2 pathway may represent a novel candidate for the treatment of diet-related metabolic derangements, with potential for EMA- and FDA-approved Jak inhibitors to be repurposed for the treatment of type 2 diabetes and/or its complications.

EXTRACELLULAR NICOTINAMIDE PHOSPHORIBOSYLTRANSFERASE (ENAMPT) BOOSTS IFNγ-INDUCED MACROPHAGE ACTIVATION

(1) G. Colombo, (2) C. Travelli, (1) C. Porta, (1) A. A. Genazzani

(1) Department of Pharmaceutical Sciences, University of Piemonte Orientale, Novara, Italy
(2) Department of Pharmaceutical Sciences, University of Pavia, Pavia, Italy

INTRODUCTION: nicotinamide phosphoribosyltransferase (NAMPT) is present in two different forms in cells: an intracellular form (iNAMPT) producing nicotinamide mononucleotide, a precursor of NAD production, and an extracellular form (eNAMPT) that exerts a cytokinic activity. The mechanism of action of eNAMPT is still under debate: it is still unknown if eNAMPT explicates its actions through an extracellular enzymatic activity or whether it binds to a still unknown receptor (CCR5 or TLR4). eNAMPT has been described has a pro-inflammatory cytokine with a potential involvement in macrophage activation and migration. Macrophages are characterized by two different phenotypes, playing a central role in orchestration and resolution of inflammation: M1-pro-inflammatory macrophages and M2-anti-inflammatory macrophages. In several pathologies, there is an unbalance between these two phenotypes: M1-macrophages mainly sustains inflammatory diseases (*i.e.,* inflammatory bowel diseases, rheumatoid arthritis), while M2-macrophages may be implicated in cancer. It has been reported that eNAMPT is over-secreted by M1-macrophages after inflammatory stimuli, promoting the migratory activity, but it is also able to drive M2-polaritazion in monocytes from patients with chronic lymphocytic leukaemia. The aim of this work is to characterize the role of eNAMPT on murine macrophages, according to different stimuli and settings of inflammatory *milieu*.
MATERIALS AND METHODS: peritoneal macrophages (PECs) were extracted from 8-12-weeks old C57BL/6 WT and TLR4 KO mice peritoneum, after 3% thioglicollate stimulation for 5 days. PECs were stimulated with 100 ng/ml LPS and/or 100 ng/ml IFNγ or 100 ng/ml IL-4 and/or 500 ng/ml recombinant eNAMPT (endotoxin levels below 0.05 EU). cDNAs were extracted for RT-PCR analysis and RNAseq analysis, cells were lysated for Western Blot and supernatants were collected for ELISA assay. To unravel the role of eNAMPT we have generated a monoclonal neutralizing eNAMPT antibody (called C269). The macrophage migration was evaluated with wound healing assay, creating a wound into cultured cells, monitoring the time of wound closure and air pouch in mice.
DISCUSSION: first, we found that eNAMPT is over-secreted in PECs after 24 and 48h of treatment with the M1-stimulated cytokines LPS and IFNγ, which was abrogated using brefeldin or monensin, while M2-cytokine IL-4 was not able to enhance its secretion. Moreover, eNAMPT was also able to synergize with IFNγ inducing M1-polarization, overexpressing M1-correlated genes (*Il1b, Il6, Il12a, Cxcl9* and *Cxcl10*) through a STAT3-dependent mechanism. According to eNAMPT ability to promote M1-polaritazion, it also stimulates migration *in vitro* and *in vivo*.
The RNA sequencing analysis has evidenced the involvement of eNAMPT on different pathways, including toll-like receptor pathway and the response to LPS, confirming the possible binding to TLR4. Importantly, the eNAMPT neutralizing antibody is able to recue eNAMPT mediated M1 polarization.
CONCLUSIONS: taken together, these data demonstrated a role of eNAMPT as a modulator of murine macrophage polarization. We have proved for the first time that, eNAMPT is a primer to IFNγ response, indeed, eNAMPT is able to significantly increase gene associated to IFNγ response, correlated to the inflammatory process. Its neutralization, with a specific antibody generated by our group, inhibits macrophage activation, as a possible therapeutic weapon in inflammatory diseases, which pathogenesis is supported by macrophage unbalance.

ASSESSING THE IMPLEMENTATION PHASE OF ADHERENCE TO BIOLOGIC DISEASE-MODIFYING ANTI-RHEUMATIC DRUGS IN TUSCAN RHEUMATOID ARTHRITIS PATIENTS THROUGH TRAJECTORIES

(1) I. Convertino, (2) S. Giometto, (3) R. Gini, (4) M. Cazzato, (2) M. Fornili, (1) G. Valdiserra, (1) E. Cappello, (1) S. Ferraro, (3) C. Bartolini, (3) O. Paoletti, (2) S. Tillati, (2) L. Baglietto, (5) G. Turchetti, (5) L. Trieste, (5) V. Lorenzoni, (1, 6) C. Blandizzi, (4) M. Mosca, (2) E. Lucenteforte, (1, 6) M. Tuccori

(1) Unit of Pharmacology and Pharmacovigilance, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
(2) Unit of Medical Statistics, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
(3) Tuscan Regional Healthcare Agency, Florence, Italy
(4) Unit of Rheumatology, University Hospital of Pisa, Pisa, Italy
(5) Institute of Management, Scuola Superiore Sant'Anna, Pisa, Italy
(6) Unit of Adverse Drug Reactions Monitoring, University Hospital of Pisa, Pisa, Italy

BACKGROUND: disease control in rheumatoid arthritis (RA) is achieved through medical monitoring evaluating adherence to disease modifying anti-rheumatic drugs (DMARDs). Adherence trajectory models may provide interesting information about the utilization patterns. This study is aimed at identifying and characterizing trajectories of the implementation phase of adherence to biologic (b) DMARDs.
METHODS: the Pathfinder study (EUPAS29263) is a retrospective population based study, using data collected in the Tuscan administrative databases. For the drug-utilization evaluation, we selected RA patients by means of a validated algorithm, which includes the first supply of biologic DMARDs from 2010 to 2015 (index date) AND a RA specialist visit in the year preceding the index date. We observed bDMARD users for 3 years or until death, neoplasia, or pregnancy. We evaluated adherence quarterly through the Medication Possession Ratio. In the first-phase analysis, we identified adherence trajectories and described the baseline characteristics. In the second-phase analysis, we focused on the trajectory most populated and we distinguished the related sub-trajectories. We performed a sensitivity analysis in both phases by using the Proportion of Days Covered for estimating adherence.
RESULTS: we identified 952 RA patients (74.8% females, mean age 52.7 years old) with a first supply of bDMARD in Tuscany.

The bDMARDs were distributed as followed: etanercept (40.7%), adalimumab (24.5%), abatacept (9%), certolizumab pegol (8.35), golimumab (6.9%), tocilizumab (6.7%), infliximab (3.9%). Among 935 users with at least three adherence values, we identified 49 fully-adherent users, 829 continuous users, and 57 early-discontinuing users. Significant differences were observed among the index bDMARDs with the exception for adalimumab and tocilizumab. The sensitivity analysis showed a decline in the mean adherence trend over time and confirmed a significant difference for golimumab. After focusing on the continuous users, three sub-trajectories were identified: continuous-steady users (556 patients), continuous-alternate users (207 patients), and continuous-declining users (66 patients). No relevant differences emerged at the baseline. The sensitivity analysis confirmed the three sub-trajectories.
CONCLUSIONS: the majority of first ever bDMARD Tuscan users showed a continuous adherence behavior in RA. The role of potential predictors of adherence trajectories and the association with effectiveness and safety outcomes should be explored by further studies.

ATOMIC FORCE MICROSCOPY AS INNOVATIVE ASSAY FOR THE MEASUREMENT OF INFLIXIMAB IN SERUM SAMPLES

(1) D. Curci, (1) M. Lucafò, (2) P. Parisse, (2) L. Casalis, (1) M. Bramuzzo, (1, 3) G. Decorti, (4) G. Stocco

(1) Institute for Maternal and Burlo Garofolo Child Health I.R.C.C.S, Trieste, Italy
(2) Elettra Sincrotrone Trieste, Trieste, Italy
(3) Department of Medical, Surgical and Health Sciences, University of Trieste, Trieste, Italy
(4) Department of Life Sciences, University of Trieste, Trieste, Italy

BACKGROUND: inflammatory Bowel Disease (IBD) are chronic, relapsing disorders with progressive bowel damage. Infliximab (IFX) is a highly effective biologic in IBD, but despite its efficacy, 20–40% of patients do not respond to treatment and 10% develop antibodies to IFX. Increasing evidences suggest that treatment failure may be associated with inadequate blood drug levels. In recent years, the introduction of biosensors based on different nanostructured materials and techniques for the rapid quantification of drugs has been proposed for therapeutic drug monitoring (TDM). In this context, we aimed to apply an assay, based on Atomic Force Microscopy (AFM) for the measurement of IFX concentration in serum samples of healthy donors and pediatric IBD patients.
METHODS: the AFM-based nanoassay measured the height signal variation of a nanostructured gold surface covered with a self-assembled monolayer of alkanethiols. In order to avoid unspecific protein adsorption, alkanethiols were terminated with oligoethylene glycol. Inside this monolayer we embedded proteins (DNA conjugated with tumor necrosis factor alpha) able to recognize the target present in solution (IFX). The system was initially fine-tuned by testing IFX concentration (0, 20, 30, 40 and 50 nM) in buffer (Tris 10 mM, EDTA 1 mM, NaCl 1 M) and then in sera of healthy donors, spiked with known concentrations of IFX within the therapeutic range (0, 10, 20, 30, 40 and 50 nM), and pediatric IBD patient enrolled at the Gastroenterology department of the Pediatric Clinic of IRCCS Burlo Garofolo in Trieste.
RESULTS: to fine-tune the AFM assay, several concentrations of IFX (0, 20, 30, 40 and 50 nM) were initially tested in buffer. A good correlation between height variation and drug concentration was found (linear regression: R^2 = 0.97 and p-value = 0.0030). The same trend was also observed in healthy donors (R^2 = 0.93 and p-value = 0.002). The sera of pediatric IBD patients treated with IFX, whose serum concentration has been already determined by the Promonitor (Grifols, Spain) ELISA gold standard assay (0, 6.7, 13.4, 20, 28 and 65 nM), were then tested by AFM. We observed a good correlation between height variation and drug concentration (R^2 = 0.98 and p-value = 0.0002) but a non significant increase in height variation was observed in pediatric patients compared to healthy donors and to the measurements performed in buffer.
CONCLUSIONS: although the present study has some limitations regarding the small sample size, we successfully demonstrated the promising use of AFM nanoassay as a potential sensitive tool for TDM for pediatric IBD patients in treatment with IFX**.** Further analysis will be necessary for the validation of AFM technique, in view of its possible application in clinical practice.

PALMITOYLETHANOLAMIDE/BAICALEIN REGULATES THE ANDROGEN RECEPTOR SIGNALING AND NF-κB/NRF2 PATHWAYS IN BENIGN PROSTATIC HYPERPLASIA

(1) R. D'Amico, (1) R. Fusco, (1) D. Impellizzeri, (1) T. Genovese, (2) M. Cordaro, (1) R. Siracusa, (3) E. Gugliandolo, (1) A. Peritore, (1) L. Interdonato, (3) R. Crupi, (1) S. Cuzzocrea, (1) R. Di Paola

(1) Department of Chemical, Biological, Pharmaceutical, and Environmental Science, University of Messina, Messina, Italy
(2) Department of Biomedical and Dental Sciences and Morphofunctional Imaging, University of Messina, Messina, Italy
(3) Department of Veterinary Science, University of Messina, Messina, Italy

BACKGROUND: benign prostatic hyperplasia (BPH) is the most common benign tumor in males. Androgen/androgen receptor (AR) signaling plays a key role in the development of BPH; its alterations cause an imbalance between prostate cell growth and apoptosis. Furthermore, chronic inflammation and oxidative stress, which are common conditions in BPH, contribute to disrupting the homeostasis between cell proliferation and cell death. With this background in mind, we investigated the effect of ultramicronized palmitoylethanolamide (um-PEA), baicalein (Baic) and co-ultramicronized um-PEA/Baic in a fixed ratio of 10:1 in an experimental model of BPH.

METHODS: BPH was induced in rats by daily administration of testosterone propionate (3 mg/kg) for 14 days. Baic (1 mg/kg), um-PEA (9 mg/kg) and um-PEA/Baic (10 mg/kg) were administered orally every day for 14 days.
RESULTS: this protocol led to alterations in prostate morphology and increased levels of dihydrotestosterone (DHT) and of androgen receptor and 5*a*-reductase expression. Moreover, testosterone injections induced a significant increase in markers of inflammation, apoptosis and oxidative stress. Our results show that um- PEA/Baic is capable of decreasing prostate weight and DHT production in BPH-induced rats, as well as being able to modulate apoptotic and inflammatory pathways and oxidative stress.
CONCLUSIONS: these effects were most likely related to the synergy between the anti-inflammatory properties of um-PEA and the antioxidant effects of Baic. These results support the view that um-PEA/Baic should be further studied as a potent candidate for the management of BPH.

GILZ CONTROLS HEMATOPOIETIC STEM CELL PROLIFERATION IN EXPERIMENTAL MODEL OF INFLAMMATION

(1) V. de Barros Z, (1) S. Bruscoli, (1) S. Flamini, (1) M. Febo, (1) M. Paglialunga, (1) G. Migliorati, (1) C. Riccardi, (1, 2) O. Bereshchenko

(1) Department of Medicine, University of Perugia, Perugia, Italy
(2) Department of Philosophy, Social Sciences and Education, University of Perugia, Perugia, Italy

INTRODUCTION: the maintenance of hematopoietic stem cells (HSC) is linked to their quiescent state, while HSC proliferation is associated with differentiation and a loss of long-term stem cell potential. The balance between HSC quiescence and proliferation is tightly regulated by intrinsic and extrinsic cues in the bone marrow. Pro-inflammatory stimuli trigger HSC proliferation to ensure adequate production of leukocytes, however with negative impact on HSC maintenance. In fact, stimulation of mice with lipopolysaccharide (LPS) that mimics bacterial infection in sepsis is associated with enhanced HSC cycling and an expansion of phenotypic HSC associated with a functional decrease in the long term. Endogenous glucocorticoid hormones (GC) regulate HSC homing via control of CXCR4 expression. Glucocorticoid-Induced Leucine Zipper (GILZ) is a gene rapidly induced by GC. It mediates many of GC' anti-proliferative and anti-inflammatory effects in several cell types. GILZ was found to limit LPS-triggered lethality in the mouse model of sepsis. The role of GC and GILZ in the control of HSC proliferation and function at steady state and upon LPS challenge is not yet defined.
METHODS: we have addressed the role of GILZ in HSC homeostasis using age-matched WT, GILZ knock-out (KO) and transgenic mice overexpressing GILZ (GILZ-TG) mice. Sepsis was achieved by LPS challenge through intraperitoneal injection of 35 µg to 50 µg of LPS, corresponding to 35 to 50 CFU (*Escherichia coli* 011:B4), 48 hours before collection of bone marrow samples. HSC frequency, number and proliferation status was evaluated using flow cytometry; differential gene expression was analyzed by RNASeq and qPCR methods; statistical analysis were performed using GraphPad Prism.
RESULTS: we found that GILZ mRNA is expressed at higher levels in HSC compared to myeloid progenitors. At steady state, young GILZ-KO mice did not show alteration in HSC number and lineage commitment compared to WT mice. However, competitive transplantation studies revealed a significant decrease in the frequency and number of GILZ-KO compared to WT HSC, suggesting that GILZ-deficient HSC have competitive disadvantage compared to WT cells. RNA-seq analysis of gene expression revealed that several cellular pathways implicated in HSC function were deregulated upon GILZ deletion. Importantly, Gene Set Enrichment Analysis showed a significant depletion of the HSC signature and an enrichment of the mTOR pathway signature in GILZ-deficient compared to WT HSC. Consistently, GILZ-deficient HSC showed enhanced proliferation as revealed by flow cytometry analysis of Ki67 expression and DNA content. As expected, LPS treatment stimulated HSC proliferation and differentiation in WT mice, and this effect was counteracted by the pre-treatment of mice with Dexamethasone. Interestingly, GILZ overexpression in GILZ-TG mice prevented the LPS-induced HSC expansion in GILZ-TG mice. Overall, these data identify GILZ is a novel regulator of HSCs function in controlling inflammation-induced HSC proliferation.
CONCLUSIONS: overall, these data identify GILZ is a novel regulator of HSCs function in controlling HSC quiescence and inflammation-induced HSC proliferation.

OLIVE LEAF EXTRACT, FROM *OLEA EUROPAEA L.*, REDUCES PALMITATE-INDUCED INFLAMMATION VIA REGULATION OF MURINE MACROPHAGES POLARIZATION

P. De Cicco, M. Maisto, G. C. Tenore, A. Ianaro

Department of Pharmacy, School of Medicine, Federico II University of Naples, Naples, Italy

BACKGROUND: obesity is one of the main threats to global human health and life expectancy. It has been accepted that obesity coincides with a low-grade inflammatory state that mediates insulin resistance and it is closely related to the pathogenesis of obesity-associated diseases. Macrophages are primarily responsible for the inflammatory response into obese adipose tissue. In particular, two different macrophage populations have been found in adipose tissue: the classically activated macrophages (M1), that promote metabolic inflammation, and the alternatively activated M2 macrophages that attenuate obesity-induced inflammation. During weight gain macrophages undergo a "phenotypic switch" from an anti-inflammatory M2 phenotype to a pro-inflammatory M1 state, which contribute to insulin resistance. Natural products provide abundant resources for anti-inflammatory compounds with potential benefits for obese patients. Olive tree (*Olea europaea L.*) leaves represent a rich sources of bioactive molecules with several beneficial effects for human health. Recently, the ef-

fect of olive leaf extract in obesity has been studied. However, the molecular mechanism in preventing obesity-related inflammation has not been elucidated.
METHODS: in the current study, we explored *Olea europaea L*. leaf extract (OLE) anti-inflammatory activity using an in vitro model of obesity-induced inflammation obtained by stimulating murine macrophages RAW 264.7 with high dose of the free fatty acid palmitate (0.5 mM), to mimic the concentration of circulating free fatty acids in obesity.
RESULTS: we found that *OLE* significantly suppressed the induction of pro-inflammatory mediators, tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, nitric oxide (NO), prostaglandin E2 (PGE2) and reactive oxygen species (ROS), while it enhanced the anti-inflammatory cytokine, IL-10. Moreover, we demonstrated that *OLE* reduced the oxidative stress induced by palmitate in macrophages by regulating the NF-E2-related factor 2 (NRF2)-Kelch-like ECH-associated protein 1 (KEAP1) pathway. Finally, we showed that *OLE* promoted the shift of M1 macrophage toward less inflammatory M2-cells via the modulation of the associated NF-κB and proliferator-activated receptor gamma (PPARγ) signaling pathways.
CONCLUSIONS: these findings shed light on the potential therapeutic feature of *OLE* in recovering obesity-associated inflammation via regulating M1/M2 status. Thereby, polyphenols from olive leaves may be used as dietary supplementation for the prevention and treatment of obesity-associated inflammation and related comorbidities.

PHARMACOLOGICAL TARGETING OF CYCLOOXYGENASE TO COUNTERACT METAFLAMMATION IN AN IN-VIVO MODEL OF DIET-INDUCED METABOLIC DERANGEMENTS

(1) G. Einaudi, (2) G. Ferreira Alves, (2) D. Collotta, (3) R. Mastrocola, (3) F. Penna, (3) C. Fornelli, (3) E. Aimaretti, (3) M. Aragno, (1) M. Bertinaria, (1) C. Cifani, (4) M. Collino

(1) Pharmacology Unit, School of Pharmacy, University of Camerino, Camerino, Italy
(2) Department of Drug Science and Technology, University of Turin, Turin, Italy
(3) Department of Clinical and Biological Sciences, University of Turin, Turin, Italy
(4) Rita Levi Montalcini Department of Neurosciences, University of Turin, Turin, Italy

BACKGROUND: in patients with metabolic diseases a low-grade chronic inflammation, known as "metaflammation", is deleteriously contributing to body weight gain, dyslipidaemia, insulin resistance and impaired glucose tolerance. Targeting proinflammatory pathways may represent an interesting therapeutic strategy to reduce the metabolic damage. Among them, cyclooxygenases (COX) are enzymes that catalyses the conversion of arachidonic acid to prostaglandins starting a cascade that leads to inflammation. COX are well-known target of drugs in inflammation-based pathologies. The aim of this study was to investigate the effects of etodolac, a nonsteroidal anti-inflammatory drug with preferable COX-2 inhibition, in an in-vivo model of diet-induced metabolic derangements.
METHODS: thirty male C57BL/6 mice were fed with a control normal diet (ND) or a high-fat diet (HD) for 23 weeks. A subgroup of 10 mice fed high-fat diet were administered etodolac orally at the dose of 20 mg/kg starting from the 18th week of the experiment (HD + ETO).
RESULTS: mice fed HD had significantly higher body weight compared to ND-fed ones starting from the 5th week of respective dietary regiment. HD mice showed insulin resistance, seen both as reduced glucose tolerance and as altered insulin signalling in the skeletal muscle, assessed via western blot technique; these parameters were instead comparable with ND group when mice treated for 5 weeks with etodolac. HD mice showed increased plasma levels of insulin and leptin and reduced systemic concentrations of ghrelin, GLP-1 and GIP compared to ND; 5-weeks treatment with etodolac restored the levels of hormones. ETO-induced COX inhibition was associated with reduced liver damage as documented by lower levels of ALT and AST, lower steatosis and inflammatory cells infiltration in livers when compared to HD. We also observed that drug treatment reduced the diet-induced systemic and local inflammation.
CONCLUSIONS: our results demonstrate that the inhibition of cyclooxygenases contribute to counteract both systemic and local metaflammation, thus resulting as viable strategy for targeting diet-induced metabolic derangements.

ESSENTIAL ROLE OF PPARγ IN IL-33-DRIVEN PRO-TUMORAL ILC2 FUNCTIONS

(1) G. Ercolano, (2) A. Gomez-Cadena, (3) N. Dumauthioz, (3) G. Vanoni, (3) T. Wyss, (1) A. Ianaro, (3) P. Romero, (2) S. Trabanelli, (2) C. Jandus

(1) Department of Pharmacy, Federico II University of Naples, Naples, Italy
(2) Department of Pathology and Immunology, University of Geneva, Geneve; Ludwig Institute for Cancer Research Lausanne, University of Lausanne, Lausanne, Switzerland
(3) Department of Oncology UNIL CHUV, University of Lausanne, Lausanne, Switzerland

BACKGROUND: Innate Lymphoid Cells (ILCs) are the most recently identified family of innate immune cells, that are emerging as potent orchestrators of immune response. In particular, ILC2s have been reported to play a critical role in disparate inflammatory diseases including asthma, chronic rhinosinusitis and allergic rhinitis. Moreover, we and others have recently reported dominant pro-tumoral functions of ILC2 in cancer. Nonetheless, regulatory factors that dictate ILC2 activation and function remain poorly studied. Peroxisome proliferator-activated receptor gamma (PPARγ) regulates the transcription of genes associated with lipid metabolism and is expressed in different immune cells, including lymphocytes, monocytes, dendritic cells and platelets where

it mainly exerts anti-inflammatory effects. Here, we characterize the expression and functional role of PPARγ in human and mouse ILCs to assess whether PPARγ can be targeted in the context of an ILC-directed immunotherapy.
METHODS: mRNA-sequencing was performed on freshly-sorted ILCs from healthy donors (HDs) peripheral blood mononuclear cells (PBMCs). PPARγ expression was confirmed by qPCR and western blot analysis in both human and mouse ILCs. PBMCs and lymph nodes from colorectal cancer (CRC) patients were used to analyze ILC frequencies, phenotype and cytokine secretion by flow cytometry assay.
RESULTS: we found that PPARγ is selectively expressed in ILC2s in humans and in mice, acting as a central functional regulator. Pharmacologic inhibition or genetic deletion of PPARγ in ILC2s significantly impaired IL-33-induced Type-2 cytokine production and mitochondrial fitness.
Further, PPARγ blockade in ILC2s disrupted their pro-tumoral effect induced by IL-33-secreting cancer cells. Lastly, genetic ablation of PPARγ in ILC2s significantly suppressed tumor growth *in vivo*.
CONCLUSIONS: our findings highlight a crucial role for PPARγ in supporting the IL-33 dependent pro-tumorigenic role of ILC2s and suggest that PPARγ can be considered as a new druggable pathway in ILC2s to inhibit their effector functions. Hence, PPARγ targeting might be exploited in cancer immunotherapy and in other ILC2-driven mediated disorders.

ENAMPT AS PRO-INFLAMMATORY CYTOKINE IN AUTOIMMUNE GASTRITIS AND CELIAC DISEASE

(1) F. Fagiani, (2) G. Colombo, (3) A. Pasini, (3) M. Lenti, (1) M. Racchi, (3) A. Di Sabatino, (1) C. Travelli

(1) Section of Pharmacology, Department of Drug Sciences, University of Pavia, Pavia, Italy
(2) Department of Pharmaceutical Sciences, University of Piemonte Orientale, Novara, Italy
(3) Department of Internal Medicine, Fondazione IRCCS Policlinico San Matteo, University of Pavia, Pavia, Italy

BACKGROUND: the extracellular nicotinamide phosphoribosyltransferase (eNAMPT), characterized by a pro-inflammatory and cytokine-like activity, has been reported to be overexpressed and oversecreted in Inflammatory bowel disease (IBD), a group of chronic relapsing-remitting diseases of the gastrointestinal tract, whose incidence and prevalence are increasing worldwide. A correlation between enhanced levels of NAMPT and worse prognosis has been reported. Notably, preclinical data clearly demonstrated that eNAMPT neutralization significantly ameliorates colitis in animal models (Colombo G, Clemente N, Zito A, *et al*. Neutralization of extracellular NAMPT (nicotinamide phosphoribosyltransferase) ameliorates experimental murine colitis. J Mol Med (Berl) 2020;98(4):595-612). However, less is known about the role of eNAMPT as cytokine in other gastrointestinal pathologies, such as autoimmune gastritis and celiac disease. Thus, our study aims to explore the inflammatory potential of eNAMPT in intestinal organ cultures from patients with autoimmune gastritis (AIG), an immune-mediated disorder characterized by the destruction of gastric parietal cells, and celiac disease (CD), an autoimmune disorder that occurs in genetically predisposed individuals.
METHODS: to study the expression of NAMPT in AIG and CD organ cultures, we collected biopsies with Jumbo forceps during colonoscopy from healthy subjects, as well as from AIG and CD patients. First, biopsies from AIG (n = 10) and CD patients (with treated and untreated CD) (n = 10) will be processed for RT-qPCR to assess the mRNA expression of NAMPT compared to healthy controls (n = 10). Furthermore, biopsies from healthy subjects (n = 10) will be weighed and plated *in vitro* as explants and stimulated with NAMPT at different concentrations (*i.e.,* 250 and 500 ng/mL). After 24 hours, supernatants were collected for the detection of a panel of cytokines (*e.g.,* TNF-α, IL-8, IL-6, IFN-γ, TGF-β, IL-33, IL-1, IL-11) and eNAMPT, by using Bio-plex technology.
RESULTS: we found an increase in the mRNA expression of NAMPT in biopsies from patients with AIG positive for *Helicobacter pylori* infection, but not an increase in eNAMPT serum levels from such cohort of patients, thus suggesting a specific local increase in NAMPT levels not observable at systemic levels in AIG patients. Moreover, we found an increase in NAMPT mRNA expression in patients with untreated CD. Notably, patients with treated CD displayed the same NAMPT mRNA expression of NAMPT healthy controls. Finally, we observed that the exposure of human biopsies from healthy patients to eNAMPT led to an increase in the mRNA expression of some pro-inflammatory cytokines (*e.g.,* IL-8, IL-6, I IFN-γ, TGF-β, IL-33) in AIG, thereby indicating that NAMPT may play a role in the exacerbation of inflammatory processes.
CONCLUSIONS: these data indicate that eNAMPT may act a pro-inflammatory cytokine in autoimmune pathologies of the gastrointestinal tract and pave a new way to investigate as a potential therapeutic target and biomarker in gastrointestinal disorders, such as AIG and CD.

EFFECTIVENESS AND SAFETY OUTCOMES ASSOCIATED WITH DIAGNOSTIC DELAY IN A REAL-WORLD COHORT OF PATIENTS WITH CROHN'S DISEASE: DATA FROM THE TUSCAN ADMINISTRATIVE HEALTHCARE DATABASES

(1) S. Ferraro, (2) C. Bartolini, (1) I. Convertino, (3) L. Bertani, (4) S. Giometto, (1) E. Cappello, (1) G. Valdiserra, (4) S. Tillati, (4) E. Lucenteforte, (5) F. Costa, (2) R. Gini, (1) C. Blandizzi, (1, 6) M. Tuccori

(1) Unit of Pharmacology and Pharmacovigilance, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
(2) Tuscan Regional Healthcare Agency, Florence, Italy
(3) Department of Translational Research and New Technologies in Medicine and Surgery, University of Pisa, Pisa, Italy
(4) Unit of Medical Statistics, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
(5) Department of General Surgery and Gastroenterology, IBD Unit, Pisa University Hospital, Pisa, Italy
(6) Unit of Adverse Drug Reactions Monitoring, University Hospital of Pisa, Pisa, Italy

BACKGROUND: diagnostic delay (DD) in Crohn's disease (CD) and its clinical consequences are poorly investigated in the real-world setting.
OBJECTIVES: to quantify the possible DD in CD patients and to evaluate its possible impact of effectiveness and safety outcomes in CD patients with DD.
METHODS: we conducted a retrospective cohort study on real-world data extracted from administrative healthcare databases of Tuscany (Italy). We included adult patients with: a record of CD diagnosis or co-payment exemption code for CD or dispensation of oral budesonide occurred between June 1st, 2011 and June 30th, 2016. The date of the earliest of these events was the index date (ID). Patients with a look-back period (time before ID) < 5 years and follow-up < 3 years were excluded. DD was defined by a record of emergency department (ED) access or hospitalization for gastrointestinal causes in the 7-60 months (7-18 months: short DD; 19-60 months: long DD) preceding ID. Patients with an ED access or hospitalization for gastrointestinal causes recorded in the 6 months before the ID or with none of these records were considered timely diagnosed (TD). We performed survival analyses (Kaplan-Meier curves) for effectiveness outcomes (time free from the first: dispensation of azathioprine, biologic drug and ileocecal resection surgery) and safety outcomes (time free from the first: ED access and/or hospitalization for any cause) over a 3-years follow-up period. Adjusted hazard ratio (aHR) was calculated by using Cox models adjusted for age, gender and number of concomitant drugs in the 30 days before ID. Both outcomes were evaluated for dichotomous (TD and DD) and categorical variables of DD (TD, short DD and long DD).
RESULTS: among 3342 CD patients, 584 (17.5%) had a suspected DD: 220 and 364 patients had short and long DD, respectively. The effectiveness analysis showed that time-free from biologic drugs was significantly different only in subjects with long DD compared with patients TD (adjusted HR (aHR): 2.17, CI 95% 1.75-2.71). No significant aHR were found for the other effectiveness outcomes. The safety analysis revealed that patients with DD have an increased risk for safety outcomes compared with TD patients: aHR: 1.59 (CI 95%:1.44-1.75) for access to ED or hospitalizations, aHR: 1.67 (CI 95% 1.50-1.85) for ED accesses, 1.50 (CI 95% 1.33-1.70) for hospitalization.
CONCLUSIONS: about 17.5% of patients showed a DD (mostly long DD). An increased risk of treatment with biologic drug, ED accesses and hospitalizations for any cause was found for patients with DD compared with those TD.

INHIBITION OF FAK-PYK2 PATHWAY PROTECTS AGAINST ORGAN DAMAGE AND PROLONGS THE SURVIVAL OF SEPTIC MICE

(1, 2) G. Ferreira Alves, (3) E. Aimaretti, (4) G. Einaudi, (3) R. Mastrocola, (2) J. Garcia Oliveira, (1) D. Collotta, (3) M. Aragno, (5) C. Thiemermann, (2) D. Fernandes, (6) M. Collino

(1) Department of Drug Science and Technology, University of Turin, Turin, Italy
(2) Department of Pharmacology, Federal University of Santa Catarina, Florianópolis, Brazil
(3) Department of Clinical and Biological Sciences, University of Turin, Turin, Italy
(4) Pharmacology Unit, School of Pharmacy, University of Camerino, Camerino, Italy
(5) Queen Mary University of London, Centre for Translational Medicine and Therapeutics, William Harvey Research Institute, Barts and the London School of Medicine and Dentistry, London, United Kingdom
(6) Rita Levi Montalcini Department of Neurosciences, University of Turin, Turin, Italy

BACKGROUND: sepsis and septic shock are associated with high mortality and are considered one of the major public health concerns. The onset of sepsis is known as a hyper-inflammatory state that contributes to organ failure and mortality. Recent findings suggest a potential role of two non-receptor proteins tyrosine kinase, the Focal adhesion kinase (FAK) and Proline-rich tyrosine kinase 2 (Pyk2) in mediating inflammation in diseases such as endometriosis, cancer, atherosclerosis and asthma. Here we investigated the potential beneficial effects of the pharmacological modulation of the FAK-Pyk2 pathway by administering the potent reversible dual inhibitor of FAK and Pyk2, PF562271 (PF271), to septic mice.
METHODS: sepsis was induced by cecal ligation and puncture (CLP) in male, five-months-old C57BL/6 mice. One hour after the CLP or Sham procedure, animals were randomly assigned to receive PF271 (25 mg/kg, s.c) or vehicle. Twenty-four hours after surgery, organs and plasma were collected for *in-vitro* analyses. A second set of experiments was performed for survival analysis. Survival rate was assessed every 12 h over the subsequent 5 days.
RESULTS: twenty-four hours after CLP, experimental sepsis led to a systemic cytokines storm including both pro-inflammatory cytokines (TNF-α, IL-1β, IL-17 and IL-6) and the anti-inflammatory cytokine IL-10. The systemic inflammatory response was accompanied by systemic high levels of ALT, AST, creatinine and lactate, as well as a high severity score. All parameters were reversed following PF271 administration. Experimental sepsis induced a local (liver and kidney) overactivation of FAK and Pyk2, which was associated to p38 MAPK activation, leading to increased expression/activation of several pro-inflammatory markers, including the NLRP3 inflammasome complex, the adhesion molecules ICAM-1, VCAM-1 and E-selectin and the enzyme NOS-2 and Myeloperoxidase. Treatment with PF271 inhibited FAK-Pyk2 activation, thus blunting the inflammatory abnormalities orchestrated by sepsis. Finally, survival analysis revealed that PF271 significantly prolonged mice`s survival time.
CONCLUSIONS: our data show, for the first time, that FAK-Pyk2 pathway contributes to sepsis-induced inflammation and its pharmacological modulation may represent new candidate for the adjuvant treatment of sepsis, due to its potential effects in counteracting hyperinflammation, which in turn reduces organ damage and ultimately promotes long-term survival protection.

LRRK-2 INHIBITION BY PF475 TREATMENT REDUCES NEURONAL DAMAGE AND IMMUNE RESPONSE AFTER SPINAL CORD TRAUMA

A. Filippone, D. Mannino, R. Basilotta, G. Casili, I. Paterniti, S. Cuzzocrea, E. Esposito

Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy

BACKGROUND: Spinal Cord Injury (SCI) is a devastating event followed by neurodegeneration, activation of the inflammatory cascade and immune system. Dysregulated or non-resolving inflammatory processes can affect neuronal homeostasis and drive immune cells stimulation. The leucine-rich-repeat kinase 2 (LRRK2) is a gene associated with the progression of Parkinson's disease (PD), and its kinase activity was found to be upregulated after instigated inflammation of the Central Nervous System (CNS) and immune system activation. Here, we aimed to investigate the efficacy of PF-06447475, an LRRK2 inhibitor, by counteracting pathological consequences of spinal cord trauma.

METHODS: the in vivo model of SCI was induced by extradural compression of the spinal cord at T6-T8 levels, then mice were treated with PF-06447475 (2.5-5 and 10 mg/kg o.s) 1 and 6 hrs after SCI.
RESULTS: we found that PF-06447475 treatments at the higher doses (5 and 10 mg/kg) showed great abilities to reduce the degree of spinal cord tissue injury, glycogen accumulation, and demyelination of neurons associated with trauma. In addition, cytokines expression levels including interleukins (IL-1, IL-6, IL-10 and 12), interferon g (IFN- γ) - and tumor necrosis factor-α (TNF-α secreted and released by immune cells after trauma were decreased by LRRK-2 inhibitor treatments. Moreover, the accumulation of $CD4^+$ and $CD8^+$ cells throughout the spinal cord lesion site of SCI mice was reduced by PF-06447475 oral administration at the dose of 10 mg/kg.
CONCLUSIONS: taken together, our results suggest that exist a mutual correlation between LRRK2, neurodegeneration and immunity and that LRRK2 inhibition could have direct effects on the intervention of neuroinflammatory disorders**.**

EFFECTS OF DOPAMINERGIC ANTIPARKINSONIAN DRUGS ON HUMAN $CD4^+$ T LYMPHOCYTES AND THEIR RELEVANCE FOR PARKINSON'S DISEASE

(1) A. Furgiuele, (1) E. Rasini, (1) MG. Albizzati, (1) M. Legnaro, (1) A. Luini, (1, 2) F. Marino, (1, 2) M. Cosentino

(1) Center for Research in Medical Pharmacology, University of Insubria, Varese, Italy
(2) Center for Research in Neuroscience, University of Insubria, Varese, Italy

BACKGROUND: Parkinson's disease (PD) is a chronic progressive neurodegenerative disease affecting approximately 7-10 million people worldwide. The clinical course of PD inexorably results in debilitating motor and non-motor symptoms, currently treated yet unresolved. $CD4^+$ T lymphocytes increasingly emerging as triggers for neurodegeneration in PD. Indeed, $CD4^+$ T cells may acquire either neurotoxic phenotypes such as the proinflammatory T helper (Th) 1 and Th17, or neuroprotective phenotypes such as the anti-inflammatory Th2 and Treg. Thus, $CD4^+$ T cells function modulation towards neuroprotection could provide novel therapeutic strategies for PD. Dopaminergic modulation of the immune response is nowadays well established, but the possible effects of dopaminergic antiparkinsonian drugs currently in use have never been assessed so far. This study aimed to test on human peripheral $CD4^+$ T cells of healthy subjects (HS) and PD patients the effects of pramipexole, ropinirole and rotigotine, which are the main dopaminergic agonists currently used as antiparkinsonian therapeutics.
METHODS: peripheral blood mononuclear cells (PBMCs) were isolated from blood of HS and drug-naïve PD patients. Isolated PBMCs were stimulated with anti-CD3/anti-CD28 Abs (2/2 μg/ml) and cultured alone or with 0.1 μM pramipexole, ropinirole or rotigotine. Cell pellets and supernatants were collected after 48 h and tumor necrosis factor (TNF)-α, interferon (IFN)-γ and interleukin (IL)-17A were assayed by RT-PCR and ELISA. The percentage of IFN-γ-, IL-17A- and IL-4-producing $CD4^+$ T cells was assessed after 5 h incubation with PMA/ionomycin (0.01/1 μg/ml) by flow cytometry. $CD4^+$ T effector (Teff) and T regulatory (Treg) cells were subsequently isolated from PBMCs by magnetic sorting. Cell proliferation of PBMCs alone and of isolated $CD4^+$ Teff alone or in the presence of Treg cells was assessed after 120 h incubation by flow cytometry.
RESULTS: in HS, stimulation of PBMCs with anti-CD3/anti-CD28 Abs increased TNF-α, IFN-γ and IL-17A at both mRNA (on average, by 4-, 8- and 4-folds, respectively), and protein levels (from 61.4 to 1304.7 pg/mL, 5.9 to 176.9 pg/mL, and 7.4 to 84.8 pg/mL, respectively). Pramipexole, ropinirole or rotigotine reduced mRNA levels of TNF-α (by 24%, 27% and 32%, respectively), IFN-γ (by 36%, 37% and 40%) and IL-17A (by 22%, 30% and 21%, respectively) in stimulated PBMCs. TNF-α was significantly reduced by ropinirole and pramipexole (by 32% and 10% respectively), while only ropinirole significantly reduced IFN-γ by 42%. Rotigotine did not affect IFN-γ secretion, while slightly decreased TNF-α and significantly increased IL-17A secretion (by 29%). Neither pramipexole, nor ropinirole or rotigotine affected the percentage of IFN-γ-, IL-17A- and IL-4-producing $CD4^+$ T cells, as well as cell proliferation of PBMCs and $CD4^+$Teff alone or co-incubated with Treg. In cells from PD patients, all dopaminergic agonists exhibited patterns of effects comparable with those reported in cells from HS.
CONCLUSIONS: all the dopaminergic agonists tested reduced TNF-α, IFN-γ and IL-17A mRNA levels in HS and PD patients. However, only ropinirole and pramipexole also resulted in reduction of TNF-α and IFN-γ production. These results suggest that dopaminergic antiparkinsonian agents may modulate $CD4^+$ T cells function to a different extent. In view of the increasing evidence about a key role of peripheral immunity and in particular of $CD4^+$ T cells in PD development and progression, further studies are needed to assess the clinical relevance of the present findings.

REGULATION OF INFLAMMATORY AND PROLIFERATIVE PATHWAYS BY FOTEMUSTINE AND DEXAMETHASONE IN ENDOMETRIOSIS

(1) R. Fusco, (1) R. Siracusa, (1) R. D'Amico, (2) M. Cordaro, (1) A. F. Peritore, (3) E. Gugliandolo, (3) R. Crupi, (4) A. Trovato Salinaro, (5) E. Raffone, (1) D. Impellizzeri, (1) S. Cuzzocrea, (1) T. Genovese, (1) R. Di Paola

(1) Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy
(2) Department of Biomedical, Dental and Morphological and Functional Imaging, University of Messina, Messina, Italy
(3) Department of Veterinary Sciences, University of Messina, Messina, Italy
(4) Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy
(5) Multi-Specialist Institute Rizzo, Torregrotta, Messina, Italy

BACKGROUND: endometriosis is a common disease. Its pathogenesis still remains uncertain, but it is clear that cell proliferation, apoptosis and chronic inflammation play an important role in its development. This paper aimed to investigate the anti-proliferative and anti-inflammatory effects of a combined therapy with fotemustine and dexamethasone.
METHODS: endometriosis was induced by intraperitoneal injections of uterine fragments from donor animals to recipient animals. Next, the pathology was allowed to develop for 7 days. On the seventh day, fotemustine was administered once and dexamethasone was administered daily for the next 7 days. On Day 14, the animals were sacrificed, and peritoneal fluids and lesions were explanted. In order to evaluate the gastrointestinal side effects of the drugs, stomachs were harvested as well.
RESULTS: the combined therapy of fotemustine and dexamethasone reduced the proinflammatory mediator levels in the peritoneal fluid and reduced the lesions' area and diameter. In particular, fotemustine and dexamethasone administration reduced the heterogeneous development of endometrial stroma and glands (histological analysis of lesions) and hyperproliferation of endometriotic cells (immunohistochemical analysis of Ki67 and Western blot analysis of PCNA) through the mitogen-activated protein kinase (MAPK) signaling pathway. Combined fotemustine and dexamethasone therapy showed anti-inflammatory effects by inducing the synthesis of anti-inflammatory mediators at the transcriptional and post-transcriptional levels (Western blot analysis of NFκB, COX-2 and PGE2 expression). Fotemustine and dexamethasone administration had anti-apoptotic activity, restoring the impaired mechanism (TUNEL assay and Western blot analysis of Bax and Bcl-2). Moreover, no gastric disfunction was detected (histological analysis of stomachs).
CONCLUSIONS: thus, our data showed that the combined therapy of fotemustine and dexamethasone reduced endometriosis-induced inflammation, hyperproliferation and apoptosis resistance.

Ahr DELETION IN TYPE 1 DENDRITIC CELLS RESTRAINS ANTITUMOR RESPONSES

(1, 2) M. Gargaro, (1) D. Ricciuti, (1) G Mencarelli, (1) G. Scalisi, (1) G. Manni, (1) E. Padiglioni, (2) M. Colonna, (2) K. M. Murphy, (1) F. Fallarino

(1) Department of Medicine and Surgery, University of Perugia, Perugia, Italy
(2) Department of Pathology and Immunology, Washington University in St. Louis, School of Medicine, St. Louis, USA

BACKGROUND: tumor therapy continues to involve invasive procedures, nonselective cytotoxic drugs, yet several patients do not respond to existing therapies. The immune system has a key role in controlling tumor initiation and growth. Conventional dendritic cells (DCs) are immune cells critical for innate and adaptive immune responses, and they include cDC1, cDC2 and pDC subtypes. cDC1s are critical for $CD8^+$ T-cell priming early in an anti-tumor response, and this function is affected by environmental signals. The transcription factor Aryl hydrocarbon Receptor (AhR) is an environmental "sensor" of specific metabolites, both endogenous and exogenous in nature, *via* association with other cell-intrinsic transcription factors. Emerging data have shed light on an unexpected role of AhR in fostering tumor escape mechanisms. Thus, we assessed the impact of AhR deletion in cDC1 on the immune response to tumors.
METHODS: cDC1 were differentiated from C57BL/6J bone marrow cells after FLT3-L treatment for 9 days. The IRF8 binding on *Ahr* promoter in cDC1, cDC2 and pDC was determined by ChIP-seq analysis and AICEs motifs were deleted by specific single guide RNAs in CAS9 expressing cDC1. Cytokine's production in cDC1 and in cDC1-$CD8^+$ T cell co-colture was analyzed by intracellular staining. *Ahrfl/fl* mice were crossed to engineered XCR1-Cre expressing mice to selectively delete AhR in cDC1. These mice and control *Ahrfl/fl* were inoculated subcutaneously with fibrosarcoma cell line and the tumor growth was monitored daily for 30 days.
RESULTS: whole genome analysis revealed that AhR is expressed in mature cDC1 to greater extent than in cDC2 and pDCs. Indeed, *Ahr* promoter analysis revealed five AICEs (AP1-IRF Composite Elements) motifs, confirmed by ChIP-seq studies as capable of binding IRF8 in cDC1. Using an integrated retroviral reporter in cDC1, we found that only one region containing the AICE1 site conferred higher reporter activity in cDC1 as compared with cDC2 and pDC cells. Selective genome editing of this AICE1 motif by CRISPR CAS9 technology suppressed AhR expression in cDC1. Moreover, AhR deletion in cDC1 abrogated the expression of tryptophan-degrading enzymes, IDO1 and IL4i1 and strongly potentiated IL-12 and TNF-α productions. Therefore, in a cDC1-T $CD8^+$ co-culture system, we demonstrated that *Ahr* deletion in cDC1 greatly increases proliferation and IFN-γ production in OTI transgenic T cells. Notably, in an *in vivo* fibrosarcoma mouse model, single-cell RNA-seq analysis showed that AhR is highly expressed in cDC1 tumor infiltrate. Accordingly, selective AhR deletion in XCRI expressing cDC1 accelerated spontaneous immune rejection of an otherwise progressive fibrosarcoma. Analysis of TME (tumor microenvironment) revealed an increased number of cDC1 and IFN-γ producing $CD8^+$ cells, suggesting that AhR activation in cDC1 inhibits efficient $CD8^+$ T cells priming against tumor antigen *in vivo*.
CONCLUSIONS: our data demonstrate that AhR is a metabolic gatekeeper able to governs immunoregulatory functions of cross-presenting cDC1. Therefore, AhR gene editing in $XCRI^+$ cDC1 may potentiate tumor immune responses. Overall, these data point to AhR as a new immune inhibitory target in cDC1, which can be targeted pharmacologically to overcome immune tolerance and resistance to immunotherapy.

TREATMENT WITH TAT-GILZ FUSION PROTEIN IMPROVES SYMPTOMS OF DSS EXPERIMENTAL COLITIS IN MICE BY AMELIORATING INTESTINAL PERMEABILITY AND RESTORING MICROBIOME DIVERSITY

(1) M. Gentili, (2, 3) L. Hidalgo-Garcia, (2, 3) T. Vezza, (1) E. Lusenti, (1) E. Ricci, (2, 3) A. Rodriguez-Nogales, (1) C. Riccardi, (2, 3) J. Galvez, (1) S. Ronchetti

(1) Department of Medicine, Pharmacology Division, University of Perugia, Perugia, Italy
(2) CIBER-EHD, Department of Pharmacology, ibs. GRANADA, Center for Biomedical Research (CIBM), University of Granada, Granada, Spain
(3) Instituto de Investigación Biosanitaria de Granada, ibs. GRANADA, Granada, Spain

BACKGROUND: Glucocorticoid-Induced Leucine Zipper (GILZ) is an early Glucocorticoid (GC)-induced gene that mimics several anti-inflammatory effects of GCs. GCs are powerful anti-inflammatory drugs employed in a variety of inflammatory, autoimmune and neoplastic diseases. In particular, GILZ exerts its activity on immune system cells as T lymphocytes, macrophages and B cells. Several studies demonstrated the protective activity of GILZ in experimental models of inflammatory bowel diseases (IBD), but the exact mechanisms underlying its multiple effects are still under investigation. In this work, we tested the beneficial effect of the treatment with a recombinant GILZ protein, specifically the trans-activator of transcription peptide-GILZ (TAT-GILZ) fusion protein in the DSS-induced colitis model
METHODS: Dextran Sodium Sulphate (DSS)-induced colitis was induced in 8-weeks old mice (C57BL/6 strain) by 3% DSS administration in drinking water. Four days after colitis induction (acute colitis), 4 separate groups of mice (n = 8/group) were treated with PBS (control), Dexamethasone (a synthetic GC), TAT (Control), and TAT-GILZ, respectively, for 5 days. Disease Activity Index (DAI) has been recorded throughout the experiment. Before sacrifice, a FITC-Dextran assay was performed to investigate variations on intestine permeability. At the end of the experimental protocol, mice were sacrificed and luminal content was collected to perform DNA extraction and sequencing. Furthermore, colon tissue was employed to perform real-time PCR, western blotting, histology, and immunohistochemistry (IHC) analyses.
RESULTS: TAT-GILZ treatment ameliorated symptoms of the disease as demonstrated by the DAI trend, compared with controls. Histological analysis showed a lower leucocyte infiltrate and a diminished histological damage score in TAT-GILZ treated mice with respect to the controls. Furthermore, TAT-GILZ treatment restored intestinal permeability as demonstrated by the FITC Dextran assay and high Zonula Occludens-1 (ZO-1) expression analysis, with levels similar to the healthy group. RNA and protein analysis of colon extracts suggests a better reepithelization in TAT-GILZ treated mice by upregulation of CD44 and CD74 receptors, upstream of ERK phosphorylation, also supported by CCND1 overexpression. Treatment with TAT-GILZ fusion protein also affected microbiome components; sequencing of fecal microbiome showed higher alpha- and beta-diversity than the other colitic groups, comparable with the healthy group.
CONCLUSIONS: TAT-GILZ fusion protein exerts a beneficial effect on DSS colitis after the onset of the symptoms. For the first time, our study demonstrates the ability of TAT-GILZ to ameliorate gut permeability and to promote colon reepithelization by overexpressing Zo-1 and cyclin D1. This new molecular mechanism reveals part of the complex mechanism through which TAT-GILZ exerts its beneficial effects on IBD models. Furthermore, TAT-GILZ treatment helps restore microbiome diversity, a key point for the maintenance of a healthy colon. In conclusion, TAT-GILZ fusion protein is a good candidate as a pharmacological tool for colitis symptoms treatment.

ROLE OF SPHINGOSINE-1-PHOSPHATE IN SEX BIAS IN ASTHMA-LIKE DISEASE

(1) E. Granato, (1) I. Cerqua, (1) A. Rossi, (2) R. Sorrentino, (2) M. Terlizzi, (1) F. Roviezzo, (1) G. Cirino

(1) Department of Pharmacy, School of Medicine, Federico II University of Naples, Naples, Italy
(2) Department of Pharmacy (DIFARMA), University of Salerno, Salerno, Italy

BACKGROUND: Sphingosine-1-phosphate (S1P) is the final product of sphingolipid metabolism. S1P is an important immune modulator responsible for physiological cellular responses. S1P signaling has long been known to mediate lymphocyte trafficking in immunity and allergy by promoting lymphocytes migration. Recently it has been defined as an intrinsic function for S1P and its receptors in both innate and adaptive immune systems independently from immune cell trafficking. Systemic administration of S1P in female mice, without adjuvant factors, triggers a Th2 response leading to a disease closely mimicking the cardinal features of severe asthma in humans such as airway hypersensitivity, pulmonary eosinophil inflammation and high circulating levels of IgE. Since there is a clear link between immune function and expression of female sex hormones in asthma, we have investigated the role of S1P-driven immune response in sex bias in asthma-like diseases.
METHODS: male and Female BALB/c mice received systemic administration of S1P (s.c.) on days 0 and 7. In another set of experiments, mice were injected with ovalbumin (OVA s.c.) on days 0 and 7; a group of mice sensitized with OVA received a treatment whit an inhibitor of S1P signaling such as L-Cycloserine. Functional, molecular, and cellular studies were performed.
RESULTS: S1P treatment induced bronchial hyperreactivity in both male and female BALB/c mice, but this effect was enhanced in females when compared to males. S1P-induced hyperreactivity was coupled to increased lung recruitment of both mast cells and $CD4^+$ T cells only in female mice. Accordingly, S1P-treated female mice displayed higher plasma IgE levels, but not males. On the other hand, the inhibition of S1P signaling in OVA-sensitised mice reversed the airway hyperreactivity only in female mice and this was coupled to reduced lung recruitment of $CD4^+$ cells and IgE plasma level, confirming the effect of S1P in driving the Th2-immune response in female mice only.
CONCLUSIONS: the presence of an association between hormone status and allergic reactivity is well established. Only during recent years, the mechanistic involvement of sex hormones in immune reactions has been acknowledged. The finding that S1P is involved in sex bias in asthma-like features is an important issue to be taken into account to define the therapeutic approach in allergic diseases further supporting the necessity of a gender tailored therapy.

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PROTECTIVE EFFECT OF A MICRONIZED FORMULATION OF N-PALMITOYL-D- GLUCOSAMINE AND HESPERIDIN AGAINST INFLAMMATION ASSOCIATED WITH INTERSTITIAL CYSTITIS/BLADDER PAIN SYNDROME (IC/BPS)

(1) E. Gugliandolo, (2) R. Di Paola, (2) S. Cuzzocrea, (1) R. Crupi

(1) Department of Veterinary Science, University of Messina, Messina, Italy
(2) Department of Chemical, Biological, Pharmaceutical and Environmental Science, University of Messina, Italy

BACKGROUND: interstitial cystitis/Bladder Pain Syndrome (IC/BPS) is a chronic inflammatory disease characterized by visceral pain. Recently, a rat model of chronic cyclophosphamide (CYP)-induced cystitis has been developed and validated. It was shown to share strong similarities with IC/BPS (and its feline counterpart), *i.e.*, the development of persistent inflammatory response, painful behaviour, bladder oedema and focal urothelial damage. Aliamides are pain relieving and anti- inflammatory lipid compounds whose parent molecule is palmitoylethanolamide (PEA). Although much attention has been paid so far to PEA, some interesting evidence on the benefits of the aliamide N-palmitoyl-D-glucosamine (PGA) is currently being gathered.

METHODS: the aim of this study was to evaluate the effects of supplementing micronized PGA (PGAm) together with the antioxidant hesperidin to rats with chronic CYP-induced cystitis. Cystitis was induced by repetitive intraperitoneal injections of CYP (40 mg/kg every three days from day 0 to day 6). Daily oral administration of PGAm-hesperidin (3:1 ratio) was started 3 days before CYP and maintained to the end of the experiment (day 10).
RESULTS: CYP instillation caused macroscopic and histological bladder inflammatory changes, increased lipid peroxidation and lowered the pain threshold. PGAm-hesperidin decreased CYP induced bladder inflammation and oxidative stress (measured by myeloperoxidase and malondialdehyde levels, respectively), decreased the number of bladder mast cells and relieved visceral pain.
CONCLUSIONS: based on these findings and the known safety profile, PGAm-hesperidin may be a useful adjunct in the management of human IC/BPS and the related feline idiopathic cystitis.

A STRUCTURE-BASED APPROACH TO MODEL PHARMACOLOGICAL INTERVENTIONS: NEW MOLECULAR ENTITIES WITH ANTI-INFLAMMATORY PROFILES

(1) F. Fagiani, (1) M. Catanzaro, (1) E. Buoso, (2) F. Basagni, (3) E. Frost, (4) E. Corsini, (1) M. Racchi, (5) T. Fulop, (1) S. Govoni, (2) M. Rosini, (1, 6) C. Lanni

(1) Department of Drug Sciences, Pharmacology Section, University of Pavia, Pavia, Italy
(2) Department of Pharmacy and Biotechnology, University of Bologna, Bologna, Italy
(3) Department of Microbiology and Infectiology, Centre de Recherches Cliniques, Faculty of Medicine and Health Sciences, University of Sherbrooke, Sherbrooke, QC, Canada
(4) Department of Environmental Science and Policy, University of Milano, Milan, Italy
(5) Geriatric Division, Department of Medicine, Faculty of Medicine and Health Sciences, Research Center on Aging, University of Sherbrooke, Sherbrooke, QC, Canada
(6) Interuniversity Center for the promotion of the 3Rs principles in teaching and research (Centro 3R), Italy

BACKGROUND: natural products offer a rich source of therapeutics. Polyphenols are diffused in nature, and many of them display a hydroxycinnamoyl function as a recurring motif. On the other hand, diallyl sulphides are garlic-derived organosulfur compounds carrying allyl mercaptan moieties. We combined these molecular fragments into new chemical entities to produce multifunctional hybrids acting simultaneously on several targets.
We delineated the general structure–activity relationships (SAR) of the hybrids by investigating anti-inflammatory properties. The activation of the Nrf2 pathway has been investigated. Nrf2 anti-inflammatory activity has been related to the crosstalk with the transcription factor NF-κB. To evaluate the potential on anti-inflammatory processes, we tested these molecules as valuable pharmacologic tools to dissect the mechanistic connection between Nrf2 and NF-κB.
METHODS: by performing ELISA, we investigated whether the activation of the Nrf2 pathway by (pro)electrophilic compounds may interfere with the LPS-induced secretion of pro-inflammatory cytokines, during immune stimulation, in a human immortalized monocyte-like cell line (THP-1). To assess the capability of compounds to modulate the NF-κB pathway, the upstream phosphorylation of IκB, NF-κB nuclear translocation, as well as the activation of NF-κB promoter were investigated by Western Blot and luciferase assay, respectively. To validate these results, we also assessed the regulation of cytokine and chemokine release by compounds, upon LPS stimulation, by Luminex X-MAP® technology in human primary peripheral blood mononuclear cells, obtained from venous whole blood of healthy patients.
RESULTS: we found that all compounds, also in the absence of electrophilic moieties, significantly suppressed the LPS-evoked secretion of pro-inflammatory cytokines. A reduction in the release of pro-inflammatory mediators similar to that induced by the compounds was also observed after siRNA mediated-Nrf2 knockdown, thus indicating that the attenuation of cytokine secretion cannot be directly ascribed to the activation of Nrf2 signalling pathway. Moreover, all compounds, with the exception of compound 1, attenuated the LPS-induced activation of the NF-κB pathway, by reducing the upstream phosphorylation of IκB, the NF-κB nuclear translocation, as well as the activation of NF-κB promoter.
CONCLUSIONS: the compounds differently regulate Nrf2 and NF-κB intracellular pathways, thus modulating innate immune cytokine release.

WHOLE TRANSCRIPTOME PROFILING IN THALIDOMIDE SENSITIVE PEDIATRIC INFLAMMATORY BOWEL DISEASE PATIENTS

(1) M. Lucafò, (1) M. Bramuzzo, (2) L. Pugnetti, (3) M. Gerdol, (3) S. Greco, (4) M. Paci, (1) D. Curci, (4) S. Renzo, (4) P. Lionetti, (3) A. Pallavicini, (1, 2) G. Decorti, (3) G. Stocco

(1) Institute for Maternal and Child Health, IRCCS Burlo Garofolo, Trieste, Italy
(2) Department of Medical, Surgical and Health Sciences, University of Trieste, Trieste, Italy
(3) Department of Life Sciences, University of Trieste, Trieste, Italy
(4) Gastroenterology Unit, Anna Meyer Children's Hospital, Florence, Italy

BACKGROUND: thalidomide has emerged as a powerful immunomodulator in the treatment of pediatric patients with inflammatory bowel disease (IBD), refractory to standard therapies. The mechanisms by which thalidomide regulates inflammation has not been clarified yet. To identify determinants of thalidomide action in pediatric IBD, high-throughput messenger RNAs (mRNA) profiles during treatment were analysed.
METHODS: IBD patients responsive to thalidomide were enrolled. mRNA profiles from peripheral blood obtained before and after twelve weeks of treatment were determined using RNAseq. Differentially expressed genes were identified by fold change from general linear model and Wald test for paired comparisons. Qualitative comparison of differentially expressed genes was done by clustering analysis. Hypergeometric test, in search of the altered gene ontology (GO) terms in the subset of differentially expressed transcripts and ingenuity pathways analysis (IPA) were also performed. The in vitro experiments were conducted by using the monocytic cell line Thp-1; to quantify PGE2 and cAMP Elisa assays were performed.
RESULTS: ten IBD pediatric patients (mean age 13.1 years, 6 males) were enrolled. RNA-seq analysis identified 252 mRNAs deregulated after treatment, 76 of which were downregulated. The hypergeometric test and IPA analysis highlighted the altered pathways: natural killer cell signalling and crosstalk with dendritic cells, eicosanoid signalling, phagosome formation and cAMP-mediated signaling. IPA revealed that the most activated upstream regulator was IL-2 (activation z-score 2.4, $p = 1.6E-05$), already associated to thalidomide activity. The involvement of the eicosanoid pathway and cAMP-mediated signaling in the mechanism of action of thalidomide was confirmed in vitro on Thp-1 cells. In particular, treatment with 20 and 200 μM of thalidomide induced a significant decrease of PGE2 production (One-way ANOVA: $p = 0.0001$. Tukey's multiple comparison $p < 0.05$). Concerning the cAMP production, treatment with 200 μM of thalidomide induced a significant increase in cAMP production (Two-way ANOVA: $p = 0.01$; Tukey's multiple comparison $p < 0.05$).
CONCLUSIONS: thalidomide induces specific gene expression alterations, which could help to elucidate its mechanism of action in pediatric IBD patients.

THE ORPHAN NUCLEAR RECEPTOR NR2F6 A NOVEL REGULATOR OF CANCER IMMUNE RESPONSES

(1) G. Manni, (1) E. Padiglioni, (2) F. A. Greco, (2) D. Passeri, (2) F. De Franco, (1) M. Gargaro, (1) G. Mencarelli, (1) G. Scalisi, (1) D. Ricciuti, (2) R. Pellicciari, (1) F. Fallarino

(1) Department of Medicine and Surgery, Section of Pharmacology, University of Perugia, Perugia, Italy
(2) TES Pharma S.R.L, Corciano, Perugia, Italy

BACKGROUND: the development of antibodies blocking the *"inhibitory immune checkpoints"* has emerged as key strategy in cancer therapies. Recently, NR2F6 (Nuclear Receptor Subfamily-2 Group-F Member-6), an orphan nuclear receptor, whose expression is highest in immune cells, has been suggested as a novel immune checkpoint, acting as a transcriptional repressor of specific cytokines relevant for tumor rejection. The exact mode of action of Nr2f6 in regulating immune responses against tumors remains still unclear. Although the role of Nr2f6 in $CD8^+$ and $CD4^+$ T cells has been documented in cancer murine models, information is still lacking about the role of Nr2f6 in other immune cells, like dendritic cells (DCs), crucial for initiating immune responses. Based on this evidence this study aimed at evaluating the role of Nr2f6 in DCs and to identify new small molecules targeting this orphan nuclear receptor.
METHODS: dendritic cells were isolated from C57BL/6 wild-type or $Nr2f6^{-/-}$ mice and differentiated in vitro for 9 days. Conventional dendritic cells cDC1 and cDC2 were purified from dendritic cells culture by magnetic sorted column after incubation with a mix of biotin conjugated antibodies plus Streptavidin. Mouse $CD4^+$ and $CD8^+$ T cells were prepared from spleen of OT.II and OT.I mice respectively and used for in vitro cross-presentation assay. Small molecule binding to Nr2f6 was identified at TES Pharma and label-free binding assay (Enspire) has been setup as NR2F6 primary screening.
RESULTS: our data clearly show that Nr2f6 is highly expressed in conventional DCs (cDCs) and mostly in the cDC1, involved in cross-presentation of tumor-associated antigens to effector cytotoxic $CD8^+$T cells. Surprisingly, in DC/T coculture systems we found that Nr2f6 deficient cDC1 induce greater T cells proliferation than wild-type cDC1, confirming the hypothesis that the absence of Nr2f6 in cDCs reprogram these cells towards a better APC functional phenotype.
Additionally, we have recently identified new small molecule inhibitor that can specifically bind to Nr2f6 protein in cell-free assays and in cell lines overexpressing Nr2f6. In particular, this new compound can increase the production of specific cytokines including IL2 and INF-γ, when added in vitro during $CD4^+$ T cells and $CD8^+$ cytotoxic T cells stimulation.
It is worth noting that, the cDC1 pretreatment with these compounds can promote T lymphocytes to produce high levels of the cytokine IL2.
CONCLUSIONS: overall, these data reveal that Nr2f6 may have critical implications in regulating the function not only of T cells but also of selected DC subsets. Moreover, these data suggest that innovative small molecules, targeting Nr2f6 may be capable to boost both T and DCs functions, resulting in potentiation of anti-cancer immune responses.

A LONGITUDINAL EVALUATION OF THE PERIPHERAL IMMUNE PHENOTYPE IN A COHORT OF ITALIAN PARKINSON'S DISEASE PATIENTS

(1) S. Martini, (2) L. Magistrelli, (2) A. Furgiuele, (3) E. Contaldi, (4) M. Legnaro, (4) E. Rasini, (4, 5) C. Comi, (4, 6) M. Cosentino, (4, 6) F. Marino

(1) Center for Research in Medical Pharmacology, University of Insubria, Varese, Italy
(2) PhD Program in Clinical and Experimental Medicine and Medical Humanities, University of Insubria, Varese, Italy
(3) Movement Disorders Centre, Neurology Unit, Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy
(4) Center for Research in Medical Pharmacology, University of Insubria, Varese, Italy
(5) Movement Disorders Centre, Neurology Unit, Department of Translational Medicine, University of Piemonte Orientale, Novara, Italy
(6) Center for Research in Neuroscience, University of Insubria, Varese, Italy

BACKGROUND: Parkinson's Disease is a common neurodegenerative disease, affecting up to 1-2 people in 1000. Prevalence increases with age and is estimated at 1% in people over 65. The histopathologic hallmark of Parkinson's Disease is the loss of dopaminergic neurons and accumulation of α-synuclein in surviving neurons, but the underlying pathophysiology is still unclear. Inflammatory mechanisms have been suggested to play a prominent role in the development of the disease, with an imbalance of immune functions, as well as neurotoxicity caused by reactive oxygen species. Further evidence highlights the involvement of peripheral adaptive immunity in Parkinson's Disease, reporting an imbalance in T cell subpopulations and in the expression of transcriptional factors in $CD4^+$ T cells in Parkinson's Disease patients and in subjects with REM sleep behaviour disorder, which is considered a potential prodromal state in the development of Parkinson's Disease.
The aim of this work is a longitudinal evaluation of the peripheral immune phenotype modifications in a thoroughly characterized cohort of Parkinson's Disease patients, setting baseline conditions at the time of Parkinson's Disease diagnosis in drug-naïve subjects and assessing peripheral immune phenotype characteristics at baseline and during disease follow-up, once a year.
METHODS: enrolment of Parkinson's Disease drug-naïve patients started in 2014. Blood withdrawal is performed on each subject at baseline and once a year during follow-up visits. A small cohort of 19 healthy control subjects with similar sociodemographic characteristics yielded control data for comparisons between the healthy subjects' cohort versus Parkinson's Disease patients' data. Collected samples are processed at the "Center for Research in Medical Pharmacology" at the University of Insubria. Lymphocytes subpopulations and peripheral immune cells are identified via flow cytometry and lymphocytes transcription factors levels are assessed through RT-PCR.
Exclusion criteria for this study are active autoimmune diseases or a medical history of autoimmune disease and the use of immunomodulatory or immunosuppressant therapy. A complete blood count analysis with differential white blood cells count, including C-Reactive Protein and Erythrocyte Sedimentation Rate, is performed as a screening for abnormal immune system activation to avoid outliers due to intercurrent inflammatory events. All subjects are clinically evaluated by movement disorders experts and demographic and clinical parameters are monitored during follow-up visits.
RESULTS: at the time of this writing, 49 Parkinson's Disease patients are enrolled for the project, with a 2:1 male to female ratio and a mean age at baseline of 68.2 ± 8.4 years (min 48, max 81). 31 subjects completed follow- up visit V1, 27 completed follow-up visit V2, 21 completed visit V3 and 16 completed visit V4. While some subjects missed baseline or follow-up visits, all of them completed at least two visits (baseline and/or follow-up). Preliminary data on transcriptional factors analyses suggests a shift in the peripheral immune system profile during follow-up and in comparison with healthy controls, with a mean significant increase in STAT1 expression levels (driving Th1 differentiation) and a decrease of STAT4 expression levels (promoting cell- mediated immunity) between V0 and subsequent follow-up visits. STAT4 and TBX21 (which is suggested to play a role in Th1 lineage development) expression levels are also reduced in the Parkinson's Disease cohort in comparison to healthy subjects. RORC and STAT3 expression levels (playing a role in Th17 cells differentiation) are also decreased in Parkinson's Disease patients compared to healthy subjects. GATA3 and STAT6 expression levels are significantly increased in Parkinson's Disease patients in comparison with healthy subjects, but are then found to significantly decrease during follow-up visits in comparison with V0 levels.
FOXP3 expression levels (linked with Treg activity) are increased in Parkinson's Disease patients at V0 versus healthy subjects but decrease during follow-up (with a significant decrease recorded between V2 and V0, in particular).
CONCLUSIONS: as far as we know, our longitudinal study is the first of its kind and offers interesting insights in peripheral immune phenotype modifications in a cohort of Italian Parkinson's Disease patients.
Collected preliminary data so far supports the hypothesis of a shifting peripheral immune phenotype in Parkinson's Disease patients compared with healthy controls and during follow-up. Our data also offers interesting insights into possible therapy-induced immune profile modifications, whose role in affecting disease progression should be further and thoroughly investigated.

IMMUNE RESPONSE AND ENDOCRINE DISRUPTING CHEMICALS (EDCs): RACK1 AS A BRIDGE BETWEEN THE ENDOCRINE AND THE IMMUNE SYSTEMS

(1, 2) M. Masi, (1) E. Buoso, (3) V. Galbiati, (3) A. Maddalon, (3) M. Iulini, (3) M. Marinovich, (1) M. Racchi, (3) E. Corsini

(1) Laboratory of Pharmacology, Department of Drug Sciences, University of Pavia, Pavia, Italy
(2) University School for Advanced Studies IUSS, Pavia, Italy
(3) Laboratory of Toxicology, Department of Pharmacological and Biomolecular Sciences, University of Milan, Milan, Italy

BACKGROUND: the Receptor for Activated C Kinase 1 (RACK1) plays a crucial role in the immune context, since a tight correlation has been demonstrated between its expression and im-

mune cells activation via protein kinase C (PKC), resulting in the modulation of pro-inflammatory cytokines and surface markers *in vitro*, *in vivo* and *ex vivo*. We demonstrated the presence of a hormone-related regulatory element for glucocorticoids and androgens in RACK1 gene promoter that mediates its transcriptional regulation, highlighting that hormone-active substances can affect the immune response via RACK1 modulation. Endocrine disrupting chemicals (EDCs) have been linked with immune alterations due to inflammation-enhancing and immunosuppressive properties and a role for EDCs in the increased incidence of cancers, autoimmune diseases, and allergies in most industrialized countries has been hypothesized. Therefore, the aim of our study was to elucidate how EDCs interfere with the immune response and to unravel the mechanisms behind their immunological implications.
METHODS: to investigate EDCs ability to modulate RACK1 expression, human promyelocytic THP-1 cells were treated with increasing concentrations of p,p'DDT (weak AR antagonist), p,p'DDE (strong AR antagonist), nandrolone, estrogen-active compounds 17β-estradiol, 17β-estradiol-BSA, diethylstilbestrol (DES), zearalenone (ZEA), bisphenols A, AF and S (BPA, BPAF, BPS), flutamide, BAY 11-7082 (NF-κB inhibitor) and agonist G1. Luciferase reporter assay, qPCR, Western blot analysis, specific sandwich ELISA and flow cytometric analysis were performed.
RESULTS: p,p'DDT and p,p'DDE induced a significant decrease in RACK1 transcriptional activity, RACK1 expression, LPS-induced IL-8 and TNF-α production and CD86 expression. Consistent with its stronger AR antagonistic effect, p,p'DDE exerts a stronger repressor effect than p,p'DDT. On the other hand, 17β-estradiol, DES, and ZEA (through GPER activation) increased RACK1 transcriptional activity and its expression, which paralleled an increase in LPS-induced IL-8, TNF-α production, and CD86 expression all dependent on RACK1/PKCβII activation. Flutamide completely prevented DES-induced RACK1 transcriptional activity and protein expression, confirming a role for AR in RACK1 transcription regulation. Finally, while BPS displayed upregulating effects on RACK1 production and consequent cytokine release, BPA and BPAF initially downregulated RACK1 but mifepristone, flutamide and BAY 11-7082 unmasked upregulating effects and shed light on their mechanism of action.
CONCLUSIONS: a complex effect results from the activity as agonist or antagonist of hormone-active substances showing the importance of RACK1 modulation and its PKC-mediated downstream effects in the immune context. Hence, RACK1 represents a bridge between the endocrine system and the innate immune system, indicating that RACK1 could be a relevant target of steroid-active substances and EDCs and even offering the opportunity to use RACK1 as a possible screening tool for immunotoxic potential of hormone-active substances.
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L-KYNURENINE INDUCES TOLEROGENIC FUNCTIONS IN cDC2 RECEPTOR ACTIVATION

(1) G. Mencarelli, (1) D. Ricciuti, (1) G. Scalisi, (1) E. Padiglioni, (1) G. Manni, (1) K. M. Murphy, (1) M. Gargaro, (1) F. Fallarino

(1) Department of Medicine and Surgery, University of Perugia, Perugia, Italy
(2) Department of Pathology and Immunology, Washington University in St. Louis, School of Medicine, St. Louis, USA

BACKGROUND: tryptophan metabolites are pivotal messengers for immune cells interactions, and among those, l-kynurenine (l-kyn) stands out for it acts rapidly and efficiently in mediating tolerogenic activity in conventional dendritic cells (cDCs). DCs represent a heterogeneous population comprehensive of three main subsets, pDC, cDC1 and cDC2, each one exerting a specific function in immune responses and tolerance. Recently, we found that a mechanism of DC tolerance, the enzyme indoleamine 2, 3-dioxygenase 1, is selectively expressed by cDC1 but not in cDC2. How tryptophan metabolites like l-kyn influence any of these cell subtypes has not received much attention. Thus, we assessed the impact of l-kyn in spreading immune tolerance in other DC subtypes.
METHODS: dendritic cells were obtained from mouse bone marrow, either Wilde Type or conditional ones (CRISPR/Cas9). Gene expression profile was performed on DCs treated with various stimuli (LPS, l-kyn) by microarray analyses. *Ido1* promoter analysis was conducted *in silico*, and Chromatin ImmunoPrecipitation was used to determine AhR involvement, binding and interaction with RelB. Experimental autoimmune encephalomyelitis (EAE) model was induced using MOG glycoprotein and scores were taken daily up to 24 days. Immune cell infiltrates and T cell activation were evaluated by Flow cytometry analysis.
RESULTS: we found that l-kyn treatment induced *Ido1* expression in inflammatory LPS-primed cDC2, both at protein and mRNA level, in AhR-dependent manner. In addition, in these cells we observed a reduced IL-6 production, and such effect was restricted to AhR competent cDC2. By ChIP assay we demonstrated that in response to l-kyn, AhR binds κB site within IL-6 promoter. To unravel the mechanism by which l-kyn promotes IDO1 induction in cDC2 through AhR, we performed *in silico* analysis of *Ido1* promoter. We found that only two canonical AhR responsive elements, located at position +1340 bp and +1601 bp, were able to confer specific retroviral vector reporter activity in cDC2 in AhR-dependent fashion. Moreover, deletion of both elements in cDC2 with CRISPR/Cas9 system, impaired IDO1 expression. Since other members of NF-κB pathway, such as RelB, are known binding partners of AhR, we tested RelB requirement in cDC2 cells for IDO1 expression. *Relb* -/- cDC2 expressed significantly less IDO1 in comparison to WT cDC2 in response to LPS and l-kyn treatment. Indeed, this treatment favored Relb/AhR dimerization and binding to *Ido1* promoter. *In vivo* experiments were carried out to understand whether l-kyn supplementation could reduce EAE symptoms. Surprisingly, l-kyn reduced EAE scores only in AhR competent mice, suppressed IFNγ and IL-17 in $CD4^+$ T cells and increased $FoxP3^+$ regulatory T cells in CNS. Finally, we demonstrated that AhR expressed by cDCs was crucial in EAE amelioration but selective deletion of AhR in cDC2 impaired l-kyn protective effect.
CONCLUSIONS: collectively, these findings suggest that AhR deletion renders pathogenic cDC2 unresponsive to the anti-inflammatory effects of l-kyn *in vitro* and *in vivo*, resulting in increased EAE susceptibility. Therefore, l-kyn may represent a novel pharmacological treatment in autoimmune diseases such as Multiple Sclerosis.

POSITIVE ALLOSTERIC MODULATION OF Src KINASE BY THE METABOLITE SPERMIDINE

(1) G. Mondanelli, (1) C. Volpi, (1) M. Gargaro, (1, 2) E. Proietti, (3) A. Macchiarulo, (1) U. Grohmann

(1) Department of Medicine and Surgery, University of Perugia, Perugia, Italy
(2) Department of Gastroenterology and Hepatology, Erasmus MC, Rotterdam, The Netherlands
(3) Department of Pharmaceutical Sciences, University of Perugia, Perugia, Italy

BACKGROUND: polyamines (*i.e.,* putrescine, spermidine and spermine) are highly bioactive polycations capable of modulating several signaling pathways. Besides their conventional role in regulating cellular physiology and tumor growth, polyamines are currently recognized as modulators of immune and inflammatory responses. Indeed, spermidine decreases the severity of colitis as well as ameliorates the multiple sclerosis symptoms in mice. Moreover, in dendritic cells (DCs), spermidine promotes the non-enzymatic functions of indoleamine 2,3-dioxygenase 1 (IDO1), an immunoregulatory molecule endowed of both catalytic activity (*i.e.*, degradation of the essential amino acid tryptophan) and signal transducing properties (*i.e.*, induction of IDO1 and TGF-β expression).
METHODS: molecular modeling approaches, mutagenesis and co-immunoprecipitation studies were used to evaluate the key residues on Src kinase involved in the interaction with the modulator (namely, spermidine) and with the substrate (*i.e.*, IDO1).
RESULTS: prompted by the discovery that spermidine tolerogenic effects are abrogated in DCs lacking either the expression of IDO1 or the activity of Src kinase, we investigated the spermidine mode of action. We found that spermidine directly binds Src in a previously unknown allosteric site located on the backside of the SH2 domain and thus acts as a positive allosteric modulator of the enzyme. As a result, Src physically interacts and phosphorylates the substrate IDO1 and, consequently, triggers the signaling transducing events.
CONCLUSIONS: overall, this study may pave the way toward the design of novel allosteric modulators able to switch on/off the Src-mediated pathways, including those involving the immunoregulatory protein IDO1.

RELEVANCE OF CD73$^+$ EXOSOMES IN SERUM OF MELANOMA PATIENTS

(1) R. Turiello, (2) M Capone, (1) E. Morretta, (1) M.C. Monti, (2) G. Madonna, (1) A. Petrella, (2) P. A. Ascierto, (1) S. Morello

(1) Department of Pharmacy, University of Salerno, Fisciano, Salerno, Italy
(2) Istituto Nazionale Tumori G. Pascale, Naples, Italy

BACKGROUND: treatment of melanoma patients with anti-PD1 (programmed cell death protein 1) monoclonal antibodies have significantly improved the survival, but many patients still do not respond to therapy. One of the major features of melanoma cells is to escape immune responses. Therefore, understanding these mechanisms is essential to identify potential predictive markers of response to immunotherapeutic agents and evaluate therapeutic strategies in combination. Extracellular adenosine, accumulated within tumor microenvironment, potently suppresses anti-tumor immune response mediated by T cells. CD73 is the rate-limiting enzyme producing extracellular adenosine. Elevated levels of CD73 in tumor tissues are associated with poor outcome in patients with solid tumor. Several studies demonstrate that CD73 also exists in a circulating form, either soluble or expressed on exosomes. We have recently demonstrated that levels of circulating CD73 activity are associated with response to anti-PD-1 agents in patients with metastatic melanoma. In this work we evaluated the potential contribution of CD73$^+$ exosomes to immune suppression and its correlation with response to anti-PD-1 agents in patients with advanced melanoma.
METHODS: exosomes were isolated by size exclusion chromatography from serum of melanoma patients before starting treatment with nivolumab or pembrolizumab (baseline) and after 30 days of treatment. Expression of CD73 and PD-L1 has been performed by flow cytometry on beads-captured exosomes using an antibody anti-CD63. The exosomal CD73 activity has been evaluated by mass spectrometry, also in presence of a selective CD73 inhibitor, APCP, or an anti-CD73 antibody. The relative fluorescence intensity values for CD73$^+$ exosomes and PD-L1$^+$ exosomes have been correlated with clinic-pathological data of patients and response to therapy. The effect of exosomal CD73-derived adenosine has been determined by functional assays in activated human peripheral T cells, also in presence of CD73 inhibitors.
RESULTS: we found that serum-derived CD73$^+$ exosomes are enzymatically active in producing adenosine. The AMPase activity of exosomes resulted significantly reduced in presence of the selective CD73 inhibitors. The levels of CD73$^+$ exosomes at baseline were similar in patients with clinical benefits (R) to anti-PD-1 therapy compared with those without clinical benefits (NR). After 30 days of therapy the levels of CD73$^+$ exosomes resulted significantly increased in NR patients compared with baseline. Conversely, levels of PD-L1$^+$ exosomes increased in R patients group after 30 days of therapy but not in NR patient group. We also observed that exosomal CD73 significantly reduced the interferon g release from activated T cells. This effect was blocked in presence of the CD73 inhibitor APCP.
CONCLUSIONS: in summary, our data show that circulating exosomes express CD73, that is enzymatically active in producing adenosine. In line with previous published data PD-L1$^+$ exosomes resulted high in patients that respond to therapy, while the levels of CD73$^+$ exosomes increased in NR patients during treatment. CD73$^+$ exosomes significantly impair the function of activated T cells. Altogether these results suggest that CD73$^+$ exosomes could limit the therapeutic efficacy of anti-PD-1 agents, contributing to suppress T cell functions.

THE COMPLICATED INVOLVEMENT OF NICOTINAMIDE PHOSPHORIBOSYLTRANSFERASE (NAMPT) IN TUMOUR MICROENVIRONMENT

M. Moro, G. Colombo, A. A. Genazzani, A. Grolla

Department of Pharmaceutical Science, University of Piemonte Orientale, Novara, Italy

BACKGROUND: nicotinamide phosphoribosyltransferase (NAMPT) exists in two distinct forms: an intracellular form (iNAMPT), which is a key enzyme involved in NAD biosynthesis, and an extracellular form (eNAMPT) that acts as a cytokine-like protein. The secreted form is released by several cell types (e.g., cancer and immune cells) and exerts a variety of biological effects. eNAMPT is reported to: (i) induce endothelial angiogenesis by promoting endothelial cell proliferation and capillary-tube formation, (ii) impact on EMT modulation in breast cancer cell line, (iii) acts as immune suppressive agent in tumour microenvironment. iNAMPT and eNAMPT are both associated with monocyte recruitment and migration, and macrophage polarization. Moreover, NAMPT negatively regulates CXCR4 transcription leading to the mobilization of suppressor myeloid populations, (iv) eNAMPT has also been linked with cancer, indeed, its serum and plasma levels are increased in several cancer patients and in most of the cases they correlate with the stage of cancer progression.
METHODS: our model provides a platform to investigate the role of eNAMPT in a mouse mammary carcinoma. 4T1 triple negative cells were engineered to express the murine NAMPT fused to the immunoglobulin signal peptide (SP-NAMPT), leading to a massive and constitutive release of NAMPT in the extracellular milieu compared to the control (SCR). This cell-modelwas used to inject BALB/c female mice to investigate tumour growth formation, neo-vessel formation, tumour-metastasization potential and the inflammatory context, focusing on T cell activation.
RESULTS: taking advantage of the histochemical analysis of tumour slices, cytofluorimetry and western blot analysis, data suggest an increase of neovascularization in SP-NAMPT tumours given by a significantly higher number of $CD31^+$ (endothelial) and $NG2^+$ (pericyte) cells. Tumour volume analysis reveala smaller growth of SP-NAMPT modelaccompaniedby an increase in metastatization potential. Metastasis were monitored in lung, brain, and liver tissues. These data suggest a direct activity of eNAMPT in tumour reprogramming. Moreover, we investigate also the inflammatory counterpart in the tumour context. Cytofluorimetric assay shows an increase in $CD4^+$ cells in SP- NAMPT tumour accompanied by an increase of $CD3^+$ $CD25^+$ cell activation (monitored by $CD73^+$ expression).
CONCLUSIONS: pericytes are known to be involved in immune regulation of both innate and adaptive immunity. They display high levels of PD-L1 and PD-L2 negatively influencing $CD4^+$ T cell activation and proliferation. Our study shows an increase of regulatory T cell activation in SP- NAMPT tumours suggesting a possible activity of pericytes on the modulation of suppressive immunity. This type of immune regulation reflects on an increased number of metastasis suggesting an aggressive phenotype of tumours correlated with a higher eNAMPT release.

GILZ, Bcl-xL AND CASPASE-8 ARE LINKED IN A NOVEL GLUCOCORTICOID-DEPENDENT APOPTOTIC PATHWAY IN THE THYMUS OF MICE

(1) I. Muscari, (1, 2) S. Adorisio, (1) L. Cannarile, (1) E. Ayroldi, (1, 2) D. V. Delfino

(1) Department of Medicine and Surgery, University of Perugia, Perugia, Italy
(2) Foligno Nursing School, Department of Medicine, University of Perugia, Foligno, Perugia, Italy

BACKGROUND: apoptosis is an essential process underlying multicellular organism development and function. In the immune system, apoptosis is required for lymphocyte development and homeostasis. In thymus physiology about 90% of thymocytes die, leaving only 10% to complete their maturation and migrate out towards peripheral lymphoid organs to mount a functional immune response. The intrinsic cell death pathway is activated by a variety of apoptotic stimuli, such as genomic toxicity and cytokine withdrawal. Bcl-2 family members control mitochondrial membrane integrity and are the major mediators in the intrinsic cell death pathway. A large body of work has suggested a critical role for Bcl-xL in protection of thymocytes from apoptosis. In the thymus, glucocorticoids (GCs) decrease Bcl-xL expression by activating signal transducer and activator of transcription 5B (STAT-5B) and recruiting it to the Bcl-xL promoter. GCs decrease Bcl-xL expression also through the transcription of glucocorticoid-induced leucine zipper (GILZ), a protein upregulated by GCs in the thymus.
METHODS: Lck^{pr}-bcl-xL transgenic mice bred on the C57BL6/J background constitutively overexpressing Bcl-xL within all thymocyte subsets were a generous gift of Dr. Craig Thompson. Female wild type (WT) or lckpr-bcl-xL transgenic (TG) mice of 4-8 weeks of age were sacrificed by cervical dislocation, thymi were excided, thymocytes were reduced to single cell suspencion and counted with a hemocytometer. Single cell suspensions were cultured at a concentration of 3×10^6 with or without 10^{-7} M dexamethasone (DEX). Total proteins were extracted and probed in Western blot experiments.
RESULTS: DEX increased the expression of GILZ in WT thymocytes but, surprisingly, the expression of GILZ in TG transgenic thymocytes was decreased irrespective of their culturing with or without DEX. Thus, Bcl-xL overexpression decreased the expression of the GC-induced protein GILZ.
Moreover, caspase-3 was not activated in untreated WT and TG thymocytes. The addition of DEX induced the activation of caspase-3 in control thymocytes; however, DEX treatment did not induce activation of caspase-3 in Bcl-xL TG thymocytes. DEX-dependent activation of caspase-8 was completely abolished in Bcl-xL TG thymocytes. This was not the case when caspase-9 activation was probed: caspase-9 activation was not inhibited by DEX in Bcl-xL transgenic thymocytes, suggesting that in thymocytes, Bcl-xL inhibits caspase-3 activation through inhibition of caspase-8 but not caspase-9 activation.
CONCLUSIONS: although the exact mechanistic role of GCs in death by neglect is currently unclear, their repression of Bcl-xL expression, a signature of death by neglect, indicates a high probability of their involvement in the process. In conclusion, our results suggest the existence of a novel non-intrinsic/non-extrinsic thymic apoptotic pathway that links GCs, GILZ, Bcl-xL, and caspase-8 in an auto-amplifying loop. Future studies are needed to clarify molecular and functional aspects of this novel pathway.

COAGULATION DISORDERS DUE TO AUTOIMMUNE REACTION AFTER ChAdOx1 nCoV-19 AND AD26.COV2.S VACCINES: AN ANALYSIS OF EUROPEAN DATA

(1) G. Nocentini, (1) L. Cari, (1) M. Naghavi Alhosseini, (1) P. Fiore, (1) G. Migliorati, (2) S. Pierno, (3) S. Pacor, (3) A. Bergamo, (3) G. Sava

(1) University of Perugia, Department of Medicine and Surgery, Section of Pharmacology, Perugia, Italy
(2) University of Bari, Department of Pharmacy-Drug Sciences, Section of Pharmacology, Bari, Italy
(3) University of Trieste, Department of Life Sciences, Trieste, Italy

INTRODUCTION: anti-SARS-CoV-2 vaccines, which elicit an immune response against the Spike protein of SARS-CoV-2, are the primary method of combating the pandemic. Real-world studies have described that the efficacy of vaccines in preventing CoViD-19 and severe CoViD-19 disease is similar.
The involvement of viruses and, in particular, SARS-CoV-2 and its Spike protein in autoimmune diseases is well known. During Spring 2021 venous blood clots and thrombocytopenia have been described in some recipients of the virus-based vaccines ChAdOx1 nCoV-19 Covid-19 (AstraZeneca) (ChA) and Ad26.COV2.S (AD26) (Janssen). In ChA recipients, blood cloths are favored by the production of anti-platelet factor 4 (anti-PF4) antibodies determining platelet activation. The frequency of thrombosis and thrombocytopenia and their association in CoViD-19 vaccine recipients are not established. To know their frequency is urgent for knowing the benefit/risk ratio.
METHODS: we assessed the frequency of severe adverse events (SAEs) and deaths documented in the EudraVigilance European database related to thrombocytopenia, bleeding, and blood clots in recipients of ChA and AD26 vaccine versus BNT162b2 Covid-19 (Pfizer/BioNTech).
RESULTS: the frequency of SAEs and SAE-related deaths in the virus-based vaccines compared to those of BNT recipients showed: 1) a higher frequency of SAEs due to venous blood clots, haemorrhage, thromboembolic disease and arterial events, including myocardial infarction and stroke, in the virus-based vaccine recipients; 2) a corresponding higher incidence of SAE-related deaths. The high frequency of SAEs in ChA and AD26 recipients was observed in both young/adult and old people.
The frequency of SAEs and SAE-related deaths in AD26 compared to ChA vaccine recipients showed: 1) lower frequency of thrombocytopenia in AD26 recipients; 2) lower frequency of SAEs in young/adult AD26 recipients; 3) higher frequency of SAEs in elderly AD26 recipients.
Under the hypothesis that BNT does not induce SAEs and deaths, the excess of subjects with SAEs after ChA and AD26 over BNT is 2-4 SAEs per 10,000 doses associated with coagulopathy, arterial, cardiac, and nervous events, leading to about 2 deaths out of 100,000 doses in the people 18-64 years old and 2-4 deaths out of 100,000 doses in the people > 64 years.
Interestingly, most of the venous thrombotic SAEs caused by ChA and AD26 are not associated with thrombocytopenia, suggesting that thrombosis with thrombocytopenia syndrome (TTS) in the presence of anti-PF4 antibodies is not the only type of thrombosis caused by virus-based vaccines.
CONCLUSIONS: both virus-based CoViD-19 vaccines are more toxic than BNT, but the frequency of the type of SAEs in different age groups is different, suggesting that the mechanisms of toxicity of ChA overlap with those of AD26 only partially.
Our data demonstrate that blood clots in virus-based recipients are associated with thrombocytopenia rarely. Thrombosis without thrombocytopenia can be explained in two ways: 1) the thrombosis is caused by anti-PF4 antibodies that are able to activate platelets only at specific sites and do not cause thrombocytopenia; 2) the thrombosis is caused by mechanisms other than anti-PF4 antibodies.

LOSS-OF-FUNCTION MUTANT OF INDOLEAMINE 2, 3-DIOXYGENASE 1 IS CRUCIAL FOR THE *IN VIVO* MALIGNANT PROGRESSION OF B16 MELANOMA

E. Orecchini, L. Zizi, M. L. Belladonna, C. Orabona

Department of Medicine and Surgery, section of Pharmacology, University of Perugia, Perugia, Italy

BACKGROUND: indoleamine 2, 3-dioxygenase 1 (IDO1) is expressed in many human cancers, and high IDO1 expression is associated with a poor prognosis in a variety of cancer types. This observation established an important link between IDO1 and immunosuppressive mechanisms active in the tumor microenvironment, providing a strong motivation for therapeutic targeting of IDO1 as one of the central regulators of immune suppression. A variety of small molecules has been developed as catalytic inhibitors of IDO1, but they failed in clinical trials both as single agent or in combination with other immune-checkpoint inhibitors. In addition to its recognized function as enzyme, IDO1 possess a non-enzymatic activity, which was largely described in murine models. Our idea is to reinterpret the role of the immune checkpoint IDO1 by encoding its non-enzymatic activity in the tumor cell.
METHODS: at this purpose, we generated a "loss of function" (LOF) mutant of the murine IDO1 protein in the tumor cell line B16-F10 melanoma. B16-F10 cells transfected with the LOF mutant of IDO1 express the protein carrying the mutation H350A that lacks the histidine (H350) required for the catalytic activity of IDO1. The in vivo tumor progression of B16.H350A in immune-competent mice was compared with the same tumor transfected with both the wild-type (WT) IDO1 and the empty vector, as controls.
RESULTS: after 20 days post tumor challenge, B16.H350A appears to be significantly more aggressive than the tumor expressing WT IDO1, suggesting that the loss of the enzymatic activity of IDO1 can support the malignant progression of the tumor, more than its catalytic activity.
CONCLUSIONS: the relevance of the non-enzymatic activity of IDO1 in melanoma tumor growth suggests a reinterpretation of IDO1 immune checkpoint in the tumor microenvironment.

AMNIOTIC FLUID STEM CELLS-DERIVED EXTRACELLULAR VESICLES REPROGRAM DENDRITIC CELL FUNCTIONS

E. Padiglioni, G. Manni, M. Gargaro, G. Scalisi, G. Mencarelli, D. Ricciuti, V. N. Talesa, P. Puccetti, R. Romani, F. Fallarino

Department of Medicine and Surgery, University of Perugia, Perugia, Italy

BACKGROUND: in the recent years the interest in extracellular vesicles (EVs) has been rapidly emerging and providing evidence on role of EVs in mediating cell to cell communication in patho/physiological conditions. Thanks to their ability to travel through all biological fluids and transfer their contents (nucleic acid and proteins) to target cells, these lipid bilayer structures has generated substantial interest in several clinical applications. Recently it has been shown that human amniotic fluid stem cells (HAFSCs) are able to release EVs (*f*HASC-EVs) with important proliferative, regenerative and immunomodulating potentials. Although the immunoregulatory functions of EVs is well documented in different preclinical models of autoimmune diseases, the mechanism by which EVs exert their effects is still poorly understood. Dendritic cells (DCs), professional antigen presenting cells (APCs), are critical regulators of innate immunity and are involved both in initiating immunity and regulating immune responses. DC functions depend not only on diversity of DC subsets (cDC1, cDC2 or pDCs) but also on specific signals from the environment. Based on this evidence, we aimed at demonstrating HAFSC-EVs impact on the function of different DC subsets *in vitro* and a model of multiple sclerosis *in vivo*.
METHODS: EVs from HAFSCs were collected by centrifugation at 100,000 xg for 60 minutes at 4 °C. Purified EVs were used to condition mouse DCs *in vitro*. DCs were purified from bone marrow of mice by flushing tibias and femurs and cultured for 9 days with Flt3-L. Conventional type 1 (cDC1) and type 2 (cDC2) DCs were separated by magnetic sorting. Uptake of HAFSC-EVs to cDCs was evaluated by FACS and confocal microscopy analysis. The role of HAFSC-EVs as mediators of biological effects was assessed on murine cDC2 *in vitro* by measuring cytokine production in the supernatant of cells treated with HAFSC-EVs over-night. Moreover, EV-conditioned cDC2 were administrated in female C57B/L6 mice immunized with myelin MOG 35-55 peptide to induce Experimental Autoimmune Encephalomyelitis (EAE). Mice were scored daily for clinical signs of EAE and cytokine transcripts were evaluated by Real time PCR.
RESULTS: we show that the HAFSC-EVs are preferentially internalized by cDC2 and minimally by other cDCs or other immune cells both *in vitro* and *in vivo*. Moreover, we found that cDC2 conditioned by HAFSC-EVs and activated by LPS released reduced levels of pro-inflammatory cytokines such as IL-6, IL-12 and TNF-α. In addition, cDC2 pretreated with HAFSC-EVs promoted regulatory T cells ($CD25^+Foxp3^+$) differentiation in cDC2-T co-cultures. Notably, cDC2 treated with HAFSC-EVs and transferred *in vivo* lead to significant amelioration of EAE clinical scores and suppression of autoimmune responses.
CONCLUSIONS: overall, our data show a new cellular mechanism by which heterologous -EVs, derived from human cells exert immunomodulatory effects in murine cDC2. leading to the control of an autoimmune disease.

THE ROLE OF GLUCOCORTICOID-INDUCED LEUCINE ZIPPER (GILZ) IN LIVER FIBROSIS: SUPPRESSION OF LEUKOCYTE RECRUITMENT INVOLVING THE CCR2-CCL2 PATHWAY

(1) M. Paglialunga, (1) Z. Antonio Viana de Barros, (1) M. Febo, (1) V. Iannello, (1) C. Riccardi, (1) G. Migliorati, (2) O. Bereshchenko, (1) S. Bruscoli

(1) Department of Medicine and Surgery, University of Perugia, Perugia Italy
(2) Department of Philosophy, Social Sciences and Education, Perugia, Italy

BACKGROUND: liver fibrosis (LF) confers a considerable clinical burden because of its severity since it is characterized by chronic inflammation and subsequently by the replacement of healthy tissue with scar tissue during liver repair. Several are the therapies currently in use and among them, great importance is given to glucocorticoids (GCs). GCs are the mainstay therapy for a wide spectrum of inflammatory and autoimmune diseases because of their potent anti-inflammatory effect and in case of LF their main purpose is limiting tissue damage and reducing the signals for fibrogenesis. Although the treatment of inflammatory and immune disorders with GCs is remarkably effective, their use is limited by harmful side effects. The risk of harm is particularly elevated for patients that follow long-term therapies and/or take high dosages. In recent years, a considerable alternative to GC treatment has been studied, namely the use of proteins such as Glucocorticoids-induced leucine zipper (GILZ, encoded in mice by *gilz* (alias *Tsc22d3)* gene). *Gilz* is a gene activated by GCs and is able to mimic many of their predominant effects, thus resulting in an efficient alternative in the treatment of inflammation.
METHODS: given the close correlation between inflammation and LF and the lack of the characterization of GILZ in LF, in this study we assessed the role of GILZ in the development of the pathology. We analyzed gene expression patterns, protein markers, leukocytes infiltration and liver toxicity markers in LF induced GILZ KO mice.
RESULTS: we found an increased LF development in *gilz* deficient mice (*gilz* knock-out (KO) mice). In fact, results showed an increase in CCL2 production and leukocyte infiltration at the early LF stage in the liver of GILZ KO mice compared to controls. Moreover, RNA interference-mediated *in vivo* silencing of CCL2 receptor CCR2 in gilz KO mice prevents the increased leukocyte recruitment and, consequently, hepatic stellate cells' activation in the livers, suggesting that the enhanced recruitment of leukocytes involves the CCR2-CCL2 pathway.
The correlation between LF and GILZ-CCL2 axis is confirmed by the levels of *gilz* mRNA expression in RNAseq data obtained from NASH and NAFLD patient liver sample. These results shown that tsc22d3 mRNA expression was considerably reduced in livers of patients presenting fibrosis compared to healthy controls, and that its expression inversely correlates with the expression of CCL2.
CONCLUSIONS: taken together these results give us a proof of concept that GILZ, an effector of GC anti-inflammatory activity, may represent a good strategy for the treatment of LF. In fact, the quest for novel therapeutic approaches is determinant both for the treatment of inflammatory liver diseases and for the reduction of side effects associated with GC therapies.

VALPROATE REDUCES INFLAMMATION ASSOCIATED WITH WOLFRAM SYNDROME

E. Panfili, G. Mondanelli, C. Orabona, M. L. Belladonna, M. Gargaro, F. Fallarino, U. Grohmann, M. T. Pallotta

Department of Medicine and Surgery, Section of Pharmacology, University of Perugia, Perugia, Italy

BACKGROUND: Wolfram syndrome (WS) is a rare autosomal-recessive disorder of the endoplasmic reticulum (ER) caused by mutations in the gene encoding wolframin (WFS1) and clinically characterized by insulin-dependent diabetes mellitus, optic nerve atrophy, and progressive neurodegeneration. Although there is no treatment for WS, pre-clinical studies suggest the use of small molecules targeting ER calcium homeostasis, including sodium valproate (VPA). A phase II clinical trial aimed at investigating the efficacy of VPA in the treatment of WS patients is ongoing. The aims of this study were to characterize a novel WFS1 mutation, to investigate a possible relationship between the WFS1 mutation and inflammation, and to evaluate VPA effects on this condition.
METHODS: peripheral blood mononuclear cells **(**PBMCs) from a WS patient were isolated on a Ficoll–Hypaque gradient and used for *in vitro* culture, treatment, and nucleic acid and protein purification. Cytokine profiles were analyzed in cell culture supernatants and in sera by using multiplex immunoassays and ELISA kits. Matched control subjects were used as a control.
RESULTS: our study described two novel WFS1 mutations in a WS patient, namely, c.316-1G > A (in intron 3) and c.757A > T (in exon 7). Both mutations, located in the N-terminal region of the protein, were predicted to generate a truncated and inactive form of WFS1. We found that the proband was characterized by a chronic inflammatory state with a dominance of T helper 17 (Th17)-type cells over regulatory T (Treg) lymphocytes and high levels of proinflammatory cytokines produced by PBMCs *in vitro* and detectable in the serum. Moreover, we found that VPA is capable to reduce the production of proinflammatory cytokines in the proband's PBMCs.
CONCLUSIONS: for the first time, our study described that WFS1-deficiency may impact the immune system, causing a state of chronic inflammation characterized by a systemic proinflammatory cytokine storm and justified further investigation for the use of VPA as a treatment for WS.

ROLE OF MYCOTOXINS IN MARINE ENVIRONMENT: PROTECTIVE ACTION OF A NATURAL EXTRACT IN A ZEBRAFISH EMBRYO AND LARVAE MODEL

A. Filippo Peritore, E. Gugliandolo, R. Crupi, D. di Paola, S. Cuzzocrea

Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy

Aflatoxin B1 (AFB1), a secondary metabolite produced by fungi of the genus Aspergillus, has been found among other foods also in fish feed and is thought to cause disease and mortality in aquaculture species. However, the effects of AFB1 on fish development and its associated toxic mechanism are still unclear. In the present study, we examined morphological alterations and oxidative stress in zebrafish embryos and larvae after exposure to different dosages of AFB1. In addition, the potentially protective effect of *Hericium erinaceus*, one of the most characterized fungal extracts with a focus on the nervous system, was evaluated. Treatment of embryos from 6 h post-fertilization (hpf) with AFB1 at 100 ng/mL significantly increased oxidative stress and induced malformations in six-day-old post-fertilization (dpf) zebrafish larvae. Evaluation of lethal and developmental endpoints such as hatching, edema, malformations, heart rate abnormality, and survival rate were assessed after 96 hours of exposure. *Hericium* inhibited morphological changes in larvae, as well as increased oxidative stress and lipid peroxidation. In conclusion, our study suggested how a natural extract such as *Hericium* may exert a partial role in promoting antioxidant defense systems and inhibiting lipid peroxidation in developing fish by counteracting the mechanism of AFB1 toxicity.

INTERLEUKIN-17A (IL-17A) INVOLVEMENT IN SYSTEMIC INFLAMMATION AND RELATED VASCULAR PRO-ACTIVE STATE IN A MOUSE MODEL OF $A\beta_{1-42}$-INDUCED ALZHEIMER'S DISEASE

(1) V. Vellecco, (2) F. Raucci, (2) A. Saviano, (1) G.M. Casillo, (1) M. Smimmo, (1) E. Panza, (1) E. Mitidieri, (1) R. D'Emanuele Di Villabianaca, (1) R. Sorrentino, (2, 3) A. J. Iqbal, (1) G. Cirino, (1) M. Bucci, (2) F. Maione

(1) Department of Pharmacy, School of Medicine and Surgery, Federico II University of Naples, Naples, Italy
(2) ImmunoPharmaLab, Department of Pharmacy, School of Medicine and Surgery, Federico II University of Naples, Naples, Italy
(3) Institute of Cardiovascular Sciences (ICVS), College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK

BACKGROUND: Alzheimer's disease (AD) is one of the most prevalent forms of dementia and neurodegenerative disorders, and to date, its treatment represents a significant unmet clinical need. A large body of experimental reports, including those from our research group, indicate the detrimental role of interleukin-17A (IL-17A) in AD pathogenesis. Considering that AD is commonly associated with circulating abnormalities and vascular dysfunction, we aimed to examine the systemic inflammation and related vascular pro-active state in a mouse model of $A\beta_{1-42}$-induced AD. Subsequently, we further investigated the potential beneficial effect of a neutralizing-IL-17A (IL-17Ab) therapy.

METHODS: mice were injected intracerebroventricularly (i.c.v.) with $A\beta_{1-42}$ peptide or its inactive control peptide $A\beta_{42-1}$ (3 µg/3 µl). To evaluate the protection profile of IL-17Ab, we used an intranasal (IN) injection route administering IL-17Ab (1 µg/10 µl) and its relative control isotype IgG2A (1 µg/10 µl) at 5, 12, and 19 days after $A\beta_{1-42}$ injection. In addition, the circulating Th17/Treg cells and related cytokines were evaluated concomitantly to haematological indexes, clot retraction, platelets activation and aggregation, and related vascular reactivity.
RESULTS: our results showed a stringent involvement of Th17 and Treg cells on the onset of AD. We also highlight that the systemic inflammation present in AD determines platelet activation, also affecting fibrinogen and clot retraction, thus promoting pro-thrombotic and pro-inflammatory processes. Moreover, we report that the administration of a neutralizing-IL-17A antibody rescues these phenomena normalizing the lymphocytes ratio, systemic inflammation, and vascular dysfunction that halts disease progression.
CONCLUSIONS: in summary, we demonstrate that IL-17 neutralizing antibody therapy effectively ameliorates Alzheimer's onset and progression by restoring immunity tolerance and reducing systemic inflammation. Innovatively, we also highlight that the decrease in IL-17 level heats the vascular dysfunction and platelet pro-active state typical of disease onset and progression.

GILZ INVOLVEMENT IN TLR2 DOWN-REGULATION BY GLUCOCORTICOIDS IN NEUTROPHILS

(1) E. Ricci, (2) E. Roselletti, (1) M. Gentili, (2) S. Sabbatini, (2) S. Perito, (1) C. Riccardi, (1) G. Migliorati, (2) C. Monari, (1) S. Ronchetti

(1) Department of Medicine and Surgery, Pharmacology Division, University of Perugia, Perugia, Italy
(2) Department of Medicine and Surgery, Medical Microbiology Division, University of Perugia, Perugia, Italy

BACKGROUND: Glucocorticoids (GCs) are the most widely used anti-inflammatory drugs, and as hormones control the expression of several target genes. GCs are also known as immunosuppressive drugs, but their function in neutrophils has not been fully understood yet. Glucocorticoid-induced-leucine-zipper (GILZ) is one of the most important GC-induced genes that mediates many anti-inflammatory and immunosuppressive effects of GCs. We previously demonstrated that GILZ is important for neutrophil microbicidal function and activity. In pathogen recognition by neutrophils, Toll-like 2 receptor (TLR2) plays an important role, as well as in the activation and functionality of neutrophils. Our aim was to investigate the regulation of TLR2 by GCs in neutrophils, possibly mediated through GILZ.
METHODS: wild-type (WT) and GILZ-Knock out (KO) mice were treated with a synthetic glucocorticoid, dexamethasone (DEX) by i.p. injection. Three hours later, mice were sacrificed to obtain peripheral neutrophils. In human studies, Polymorphonuclear cells (PMNs) were isolated from buffy coats. Mice and human neutrophils were used to analyze TLR2 expression by flow cytometry and neutrophil killing function by in vitro tests. Immunofluorescence analysis and in situ Proximity ligation assay were used to explore GILZ, NF-κB, and STAT5 localization and their potential interactions.
RESULTS: TLR2 was found to be down-regulated after DEX treatment in WT but not GILZ-KO peripheral neutrophils. Accordingly, the anti-fungal activity of neutrophils was reduced in WT but not GILZ-KO cells. As our data indicated the involvement of GILZ in TLR2 down-regulation, we searched for a GILZ-binding partner in the regulation of TLR2 expression. We demonstrated that GILZ binds the transcription factor STAT5, but not NF-κB, which is known to play a fundamental role in TLR2 expression. The binding of GILZ and STAT5 prevents its nuclear translocation thus limiting TLR2 transcription and expression. DEX was able to downregulate and counteract TLR2 expression in GM-CSF-activated human neutrophils, which correlated with a decrease in their anti-fungal activity. GILZ expression inversely correlated to TLR2 expression.
CONCLUSIONS: we here demonstrate that GCs, while playing a known protective role against neutrophils by preserving their survival, alter their microbicidal functions by downregulating TLR2. We show that GILZ is the key player of GC-mediated TLR2 downregulation, by virtue of its ability to bind to STAT5, thus preventing the transcription of TLR2. Therefore, GCs are considered to preserve the first line of immune defense against infections by microorganisms, but our data suggest that a GC acute treatment reduces the pathogen recognition by neutrophils with consequent reduced anti-microbicidal activity.

AHR-MEDIATED IMMUNOMODULATORY EFFECTS OF STATINS IN INFLAMMATORY BOWEL DISEASE

(1) D. Ricciuti, (1) G. Manni, (1) M. Gargaro, (1) G. Scalisi, (1) G. Mencarelli, (1) E. Padiglioni, (2) I. Monteleone, (1) P. Puccetti, (1) F. Fallarino

(1) Departement of Medicine and Surgery, Section of Farmacology, University of Perugia, Perugia, Italy
(2) Department of Biomedicine and Prevention, Tor Vergata University of Rome, Rome, Italy

BACKGROUND: statins are well-known inhibitors of 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA) that lower serum cholesterol and the risk of major vascular events. Statins are also able to activate alternative and protective pathways associated with anti-inflammatory effects. The Aryl hydrocarbon receptor (AhR), plays a pivotal role in the control of inflammatory responses. AhR is a ligand-activated transcription factor expressed in several immune system cells, such as dendritic cells (DCs), which are essential components of innate and adaptive immunity and play a crucial role in the maintenance of immune tolerance in the gut. In this study we evaluated the ability of statins to act as AhR ligands and to mediate immune regulatory properties in dendritic cells. Moreover, we assessed potential statin effects in Inflammatory Bowel Diseases (IBD) model.
METHODS: the ability of different statins to bind and activate AhR was assessed by the luciferase assay performed on murine hepatocytes overexpressing AhR and XRE (Xenobiotic responsive

elements) and murine embryonic fibroblast (MEF) transfected with various mutants of AhR. Statin effects on DCs, differentiated from C57Bl/6 bone marrows with FLT3L for 9 days, were evaluated in vitro experiments after treatment of DCs with lipopolysaccharide (LPS) in combination with statins using cytokine multiplex analysis. Ahr f/fVav iCre and Ahr f/fCD11c iCre conditional mice were employed to study AhR-mediated anti-inflammatory effects of Atorvastatin in immune cells and DCs respectively, in a TNBS-induced colitis model.
RESULTS: we found that among the different tested statins only Atorvastatin and less strongly Rosuvastatin and Pravastatin induced AhR activation, Importantly, such effect was prevented in cells carrying out specific AhR mutants. We confirmed high AhR expression in DCs that increased upon treatment with the LPS. Concomitant treatment of DCs with LPS and Atorvastatin promoted suppression of IL-6 and IFN-γ production, while increased IL-10 secretion. Notably, this effect was reverted in AhR-deficient DCs. In vivo we showed that Atorvastatin administration protected against colitis by a mechanism requiring AhR in conventional dendritic cells.
CONCLUSIONS: these data demonstrate that selected statins are a new ligand of AhR. Moreover, our findings suggest a therapeutic repositioning of Atorvastatin in the treatment of inflammatory bowel disease.

ANTI-INFLAMMATORY AND IMMUNOMODULATORY ACTIVITY OF MANGIFERA INDICA L. EXTRACT IN THE ONSET AND PROGRESSION OF GOUTY ARTHRITIS

(1) A. Saviano, (1) F. Raucci, (2) G. M. Casillo, (3, 4) A. A. Mansour, (1) A. Pernice, (2) M. Smimmo, (2) V. Vellecco, (5) V. Brancaleone, (1) N. Mascolo, (3) A. J. Iqbal, (2) M. Bucci, and (1) F. Maione

(1) *ImmunoPharmaLab*, Department of Pharmacy, School of Medicine and Surgery, Federico II University of Naples, Naples, Italy
(2) Department of Pharmacy, School of Medicine and Surgery, Federico II University of Naples, Naples, Italy
(3) Institute of Cardiovascular Sciences (ICVS), College of Medical and Dental Sciences, University of Birmingham, Birmingham, UK
(4) Department of Clinical Laboratory Sciences, College of Applied Medical Sciences, King Khalid University, Abha, Saudi Arabia
(5) Department of Science, University of Basilicata, Potenza, Italy

BACKGROUND: gouty is a paradigm of acute inflammation caused by depositing monosodium urate (MSU) crystals within articular areas. The infiltration of leukocytes drives the initial inflammatory response followed by a secondary wave of lymphocytes. In the context of inflammation, it has been reported that *Mangifera indica* L. displays a prominent anti-inflammatory activity. Therefore, we aimed to evaluate *Mangifera indica* extract (MIE, 90% in mangiferin) in a mouse model of MSU-induced gouty arthritis.
METHODS: Joint inflammation was induced in CD-1 mice by the intra-articular (i.a.) administration of MSU crystals (200 μg 20 μl^{-1}). MIE (0.1-10 mg kg^{-1}; orally (p.o.) gavage) or corresponding vehicle (DMSO/saline 1:3; p.o. gavage) were administrated concomitantly to MSU (time 0) and 6, 12 and 18 h after the stimulus, for a total of four times. Thereafter, joints were scored macroscopically, and knee joint edema was determined with a caliper. In addition, myeloperoxidase assay and western blots analysis for COX-1, COX-2/mPGES-1, mPGES-2, and mPTGDS-1/PPARγ expression were conducted at 24 h to evaluate leukocytes infiltration and activation, followed by the analysis of *in situ* and circulating pro/anti-inflammatory cyto-chemokines. Moreover, flow cytometry was used to evaluate the modulation of infiltrated inflammatory monocytes and local and systemic Th17/Treg profiles.
RESULTS: treatment with MIE (0.1-10 mg kg^{-1}, p.o.) revealed a dose-dependent reduction of joint inflammation scores and knee joint edema with maximal inhibition at 10 mg kg^{-1}. The plant extract significantly reduced MPO activity and the expression of different pro-inflammatory cyto-chemokines in inflamed tissues. Furthermore, biochemical analysis revealed that MIE modulated COX-2/mPGES-1 and mPTGDS-1/PPARγ pathways. Flow cytometry analysis also highlighted a significant modulation of infiltrating inflammatory monocytes ($CD11b^{+ve}/CD115^{+ve}/LY6C^{hi}$) and circulating Treg cells ($CD4^{+ve}/CD25^{+ve}/FOXP3^{+ve}$) after MIE treatment.
CONCLUSIONS: collectively, the results of this study report the capability of MIE to positively affect the systemic immunological perturbance beyond a prominent inflammatory reaction.

SELECTIVE DELETION OF IDO1^{+} cDC1 WORSENED CNS INFLAMMATION IN AN EXPERIMENTAL MODEL OF MULTIPLE SCLEROSIS

(1) G. Scalisi, (1) G. Mencarelli, (1) D. Ricciuti, (1) E. Padiglioni, (1) G. Manni, (2) K. M. Murphy, (1) M. Gargaro, (1) F. Fallarino

(1) Department of Medicine and Surgery, University of Perugia, Perugia, Italy
(2) Department of Pathology and Immunology, Washington University in St. Louis, School of Medicine, St. Louis, USA

BACKGROUND: Dendritic cells (DCs) are specialized antigen presenting cells, highly adapted to sense pathogens and to induce the development of adaptive immune responses. They form a complex network of phenotypically and functionally distinct subsets, including cDC1 and cDC2 conventional DCs and plasmacytoid DCs (pDCs). cDC1 and cDC2 function both in initiating immune responses against pathogens and in maintaining self-tolerance. It is known that tolerogenic cDCs, expressing the tryptophan metabolic enzyme indoleamine 2,3-dioxygenase 1 (IDO1), control inflammation trough regulatory T cell induction in an experimental autoimmune encephalomyelitis (EAE). However, the specific regulatory cDC subset lowering CNS inflammation is poorly defined.
METHODS: we first analyze the IDO1 expression in cDC subsets *in vitro* and *in vivo* by intracellular staining. The transcriptional mechanism inducing *Ido1* in cDC1 was dissected by ChIP-seq, a

GPF-retroviral vector assay and Crisp-CAS9 mediated silencing. Then we induced EAE model in *Irf8 Δ32*$^{-/-}$ mice, lacking cDC1, and WT as counterparts and analyzed the EAE symptoms. We analyzed the contribution of IDO1 in cDC1 tolerogenic activity by generating a mouse specifically lacking IDO1 in cDC1 (Ido1$^{f/f}$/Xcr1$^{+/+}$) with cre-lox recombination technology, under the cDC1 specific Xcr1 promoter.
RESULTS: first of all, we found that IDO1 is selectively expressed in mature CCR7^{+} cDC1 but not in CCR7^{+} cDC2. Interestingly, we discovered that IRF8 and BATF3, cDC1 specific transcription factors, control *Ido1* expression, through the recognition of a region containing two AP-1-IRF Composite Elements (AICEs) at - 126 bp (AICE1) and + 495 bp (AICE2) in *Ido1* promoter. In particular, only AICE1 enhancer element showed retroviral reported activity in mature cDC1 and its CAS9-mediated silencing abrogated IDO1 expression. Moreover, IDO1 confers to CCR7^{+} cDC1 a tolerogenic signature, characterized by increased PDL1 expression and inhibition of T cells proliferation.
We elucidated the role of tolerogenic IDO1^{+} cDC1 in controlling CNS inflammation. Surprisingly, the selective loss of IDO1 in cDC1 worsened EAE symptoms, promoting CNS immune cell infiltration, likewise in mice lacking cDC1. Such mice showed severe EAE symptoms, a greater CNS immune cell infiltration and increased pathogenic cytokines compared to control. On the other hand, we observed a significant decrease in PDL1 expression in Ido1$^{f/f}$/Xcr1$^{+/+}$ relative to littermate control, as well as a reduction of TGF-β and regulatory T cells frequency.
Finally, preclinic data were confirmed in single cell RNA-seq analysis of multiple sclerosis (MS) patients. Accordingly, we found that IDO1 was the only tryptophan metabolic enzyme expressed by cDC1 in cerebrospinal fluid (CSF) in relapsing remitting (RR) MS patients compared to healthy donors, but a slight IDO1 cDC1^{+} decrease was observed in RR blood.
CONCLUSIONS: these data highlight how IDO1 expressing cDC1 contribute to control EAE pathogenesis and they might be considered a potential biomarker in neuroinflammatory disease. Further investigations can help to understand whether a decrement of circulating IDO1^{+} cDC1 may correspond to a disease exacerbation in RR patients. Indeed, the activation of a cDC1 mediated tolerogenic response may represent a strategy to establish a long-term tolerance to block CNS inflammation in early MS patients and promote myelin reconstitution.

EVALUATING THE PROTECTIVE PROPERTIES OF A XYLOGLUCAN-BASED NASAL SPRAY IN A MOUSE MODEL OF ALLERGIC RHINITIS

S. A. Scuderi, M. Lanza, G. Casili, A. Filippone, M. Campolo, I. Paterniti, S. Cuzzocrea, E. Esposito

Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy

BACKGROUND: a breached nasal epithelial barrier plays an important role in driving allergic rhinitis (AR). Corticosteroids remain the standard of care (SoC) but come with side effects, thus alternative safe and effective treatments able to avoid inflammation and restore barrier integrity are needed. The aim of the present study is to evaluate the barrier-forming capacity of a xyloglucan-based nasal spray (XG) and compare its efficacy to several SoC treatments (corticosteroid spray, oral mast-cell stabilizer and oral anti-histamine) in reducing allergic responses in addition to its effect when concomitantly administered with an anti-histamine.
METHODS: an ovalbumin (OVA)-induced mouse AR model was used. Sensitization of the mice was carried out via an injection of 25 µg of OVA and 2 mg of aluminum hydroxide on days 0, 7 and 14. The mice were then subjected to intranasal challenges with 100 µg of OVA for 7 consecutive days from days 21 to 27. XG (20 µL of XG containing 0.02 µg per mouse) and saline (20 µL per mouse) were administered by intranasal instillation on days 21 to 27 (3 hours before intranasal OVA challenge). For the other groups, we evaluated and compared the efficacy of a corticosteroid, anti-histamine and mast-cell stabilizer treatment to XG-based spray, and also assessed the concomitant efficacy of antihistamines with the XG-based spray.
RESULTS: XG shows a significant efficacy in reducing histological damage in AR mice; improves nasal rubbing and histamine-induced hyper-responsiveness. Total and OVA-specific IgE as well as pro- inflammatory cytokines are significantly reduced compared to OVA challenged-mice. However, XG reduces mucous secreting cells (PAS-positive) and mucin mRNA expression similar to the corticosteroid-treated mice. XG-spray maintains tight junction protein expression (ZO-1) and conversely decreases HDAC1 significantly. Moreover, the concomitant treatment showed in all of the endpoints a similar efficacy to the corticosteroids.
CONCLUSIONS: therefore, based on the results obtained, this innovative approach may represent a novel therapeutic strategy for nasal respiratory diseases like AR, reducing undesirable side effects and improving the quality of life in patients.

HIDROX® AND CHRONIC CYSTITIS: BIOCHEMICAL EVALUATION OF INFLAMMATION, OXIDATIVE STRESS, AND PAIN

(1) R. Siracusa, (1) R. D'Amico, (2) A. Trovato Salinaro, (3) M. Cordaro, (1) R. Fusco, (1) D. Impellizzeri, (1) L. Interdonato, (2) M. Scuto, (2) M. L. Ontario, (4) R. Crea, (2) V. Calabrese, (1) R. Di Paola, (1, 5) S. Cuzzocrea

(1) Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy
(2) Department of Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy
(3) Department of Biomedical, Dental and Morphological and Functional Imaging University of Messina, Messina, Italy
(4) Oliphenol LLC., Hayward, California, US A
(5) Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, St. Louis, USA

BACKGROUND: interstitial cystitis/painful bladder syndrome (IC/PBS) is a chronic bladder condition characterized by frequent urination, inflammation, oxidative stress, and pain. The aim of the study was to evaluate the anti-inflammatory and antioxidant effects of an oral administration of Hidrox® (10 mg/kg) in the bladder and spinal cord in a rodent model of IC/BPS.
METHODS: the chronic animal model of cystitis was induced by repeated intraperitoneal injections of cyclophosphamide (CYP) for five consecutive days. Treatment with Hidrox® began on the third day of the CYP injection and continued until the 10th day.
RESULTS: CYP administration caused macroscopic and histological bladder changes, inflammatory infiltrates, increased mast cell numbers, oxidative stress, decreased expression of the tight endothelial junction (*e.g.*, zonula occludens-1 (ZO-1) and occludin), and bladder pain. Treatment with Hidrox® was able to improve CYP-induced inflammation and oxidative stress via the nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase 1 (HO-1) pathway. It was also able to reduce bladder pain which was aggravated by the activation of neuroinflammation in the central nervous system. In particular, Hidrox® reduced the brain-derived neurotrophic factor (BDNF), as well as the activation of astricites and microglia, consequently reducing mechanical allodynia.
CONCLUSIONS: these results indicate that nutritional consumption of Hidrox® can be considered as a new therapeutic approach for human cystitis, increasing the conceivable potential of a significant improvement in the quality of life associated with a lowering of symptom intensity in patients with IC/BPS.

NEW PERSPECTIVES IN HUMAN NON-SMALL CELL LUNG CANCER: IDO2 AS A POTENTIAL PROGNOSTIC BIOMARKER

(1) C. Suvieri, (2) M. Mandarano, (1) S. Rossini, (3, 4) A. Carvalho, (2) A. Sidoni, (1) U. Grohmann, (1) C. Volpi

(1) Section of Pharmacology, Department of Medicine and Surgery, University of Perugia, Perugia, Italy
(2) Section of Anatomic Pathology and Histology, Department of Medicine and Surgery, Medical School, University of Perugia, Perugia, Italy
(3) Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal
(4) ICVS/3B's - PT Government Associate Laboratory, Braga/Guimarães, Portugal

BACKGROUND: Indoleamine 2,3-dioxygenase 2 (IDO2) is less known than its paralog IDO1, a well-characterized immunomodulatory enzyme. Due to their high sequence similarities, IDO2 is often considered as endowed with a molecular function similar to IDO1, although IDO2 has a weak tryptophan catalytic activity in vitro and in vivo. Thus, the role of IDO2 in the immune response and inflammatory processes may be dependent on a distinct function. Unlike IDO1, its dysregulation in tumors is poorly documented. Nevertheless, a high prevalence of two loss-of-function of hIDO2 single nucleotide polymorphisms (SNPs; R248Wand Y359X) has been reported in various types of cancer, including non-small cell lung cancer (NSCLC), the most common lung malignancy for which an effective drug target still represents an unmet need.
METHODS: the study was approved by the Local Ethics Committee. NSCLC patients were recruited from S.M. Misericordia Hospital in Perugia. Surgical specimens were formalin-fixed and paraffin-embedded and used for immunohistochemical analysis with an hIDO2 primary antibody. The staining correlated to a standard H-Score (sum of intensity and percentage of tumor cells labeled). Hematoxylin and Eosin staining was used to determine histological subtypes (2020 WHO) and the localization/density of tumor-infiltrating lymphocytes. The genotype was evaluated in NSCLC patients and healthy control subjects. The human lung carcinoma cell line A549 was used for subcellular fractionation assay. Briefly, cells were homogenized and loaded in a continuous sucrose gradient (16-55% w/w). After ultracentrifugation, all fractions were collected and separated by SDS-PAGE for WB analysis with the IDO2 primary antibody.
RESULTS: the immunohistochemical analysis of NSCLC specimens associated to clinical-pathological data indicated that the adenocarcinoma histotype has a higher IDO2 expression ($p < 0.001$). Moreover, in this histotype, high IDO2 protein was significantly correlated with tumor-infiltrating lymphocyte intratumoral or mixed localization ($p < 0.001$). Among other molecules, high PDL1 levels were strictly related to high IDO2 expression in squamous cell carcinomas ($p = 0.012$). Remarkably, 83% of tumors showed a membrane reinforcement staining of IDO2 that, in 51% of the cases, localized at the cell membrane basolateral side between tumor and stromal tissue. In accordance with these data, preliminary subcellular fractionation analysis in A549 cells showed a higher IDO2 protein in membrane compared to the cytosolic fractions.
In a large cohort of NSCLC patients and healthy counterparts, the correlation between IDO2 SNPs and the cancer risk was also evaluated. The results showed that NSCLC patients display a significantly different R248W genotype distribution compared to the control group.
Furthermore, for the first time, we found a significant correlation between IDO2 high expression and worst NSCLC survival prognosis ($p = 0.011$).
CONCLUSIONS: the results of this study supported the hypothesis that IDO2 might play a critical role in the development and progression of tumors and, in particular, improved our knowledge in NSCLC. Indeed, IDO2 dysregulation, association with other molecules, and prognostic significance could suggest the use of selective compounds as a new cancer immunotherapeutic strategy. Further studies are needed to better clarify the IDO2 function, possibly dependent on the presence of specific factors or a possible correlation between genetic variants and activity.

UTILIZATION PATTERNS AND HEALTHCARE RESOURCES IN NEW USERS OF BIOLOGIC THERAPIES FOR ULCERATIVE COLITIS IN TUSCANY: THE MICHELANGELO STUDY

(1) G. Valdiserra, (2) S. Tillati, (3) V. Lorenzoni, (4) R. Gini, (3) G. Turchetti, (2) S. Giometto, (4) C. Bartolini, (4) O. Paoletti, (1) I. Convertino, (1) S. Ferraro, (1) E. Cappello, (1, 5) M. Fornai, (2) E. Lucenteforte, (1, 5) M. Tuccori

(1) Unit of Pharmacology and Pharmacovigilance, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
(2) Unit of Medical Statistics, Department of Clinical and Experimental Medicine, University of Pisa, Pisa, Italy
(3) Institute of Management, Scuola Superiore Sant'Anna, Pisa, Italy
(4) Tuscan Regional Healthcare Agency, Florence, Italy
(5) Unit of Adverse Drug Reactions Monitoring, University Hospital of Pisa, Pisa, Italy

BACKGROUND: biologic treatments for ulcerative colitis (UC) (adalimumab, infliximab, golimumab, vedolizumab) are recommended when failure with traditional therapies occurred. Real-world studies may provide important information for the optimization of care. The study aim is to describe the utilization patterns and the Regional Healthcare System (RHS) facilities of Tuscany in new users of UC biologic therapies.
METHODS: a descriptive, retrospective cohort study (EUPAS40896) was performed using Tuscan healthcare administrative databases, namely the hospital discharge registry, the emergency department (ED) admission records and the drug-reimbursement database. We created four drug-user cohorts with the following inclusion criteria: first supply of a biologic therapy (one cohort for each of the four drugs of interest) from 2015 to 2019 (index date); ≥ 18 years old; five years of history data (look-back period); at least one year of follow-up; UC diagnosis OR UC co-payment exemption code in the look-back or in the follow-up periods OR a gastroenterological visit in the year before the index date. We estimated in 1 year follow-up: the mean adherence to index drug (Proportion of Day Covered); the percentage of users switching to other biologic therapies and the related mean number of Described Daily Dose (DDD); survival analysis to the index drug (Kaplan Maier); the percentage of concomitant therapies per biologic users, the percentage of patients with at least one gastroenterological visit; ED access and hospitalizations for any cause per biologic cohorts; the time to the first ED access and to hospitalization for any cause.
RESULTS: the new users of biologic therapies were: 239 adalimumab, 175 infliximab, 110 golimumab, 107 vedolizumab. We observed a continuous use of biologics with a high mean adherence over the one-year follow-up, ranging from 94% to 152%. Patients switching to infliximab were 7% from adalimumab users, 11% from golimumab, and 7% from vedolizumab, and the mean number of DDD was 5813, 5145 and 1253, respectively. While, patients switching from infliximab to vedolizumab were 10% with a mean number of DDD of 3167. In the one-year follow up, > 80% of biologic users remained in treatment with the index drug among all cohorts. The most used concomitant therapies were: antibiotics (> 60% among all biologic users), mesalazine (> 60% among all biologic users) and systemic corticosteroids (> 40% among all biologic users). Patients with at least one ED access were: 36% adalimumab, 37% infliximab, 43% vedolizumab, 38% golimumab. Patients with at least one hospitalization were: 35% infliximab, 26% adalimumab, 30% golimumab, and 29% vedolizumab. The mean time to the first ED access and to the first hospitalization ranged from 140 (infliximab) to 176 days (adalimumab) and from 139 (golimumab) to 166 days (vedolizumab), respectively. Patients with at least one gastroenterological visit were: 40% adalimumab, 12% infliximab, 54% vedolizumab, 30% golimumab.
CONCLUSIONS: this study showed that first users of UC biologic therapies had high adherence to the index drugs and the occurrence of ED accesses, hospitalizations and gastroenterological visits was almost similar among all cohorts, while the time to the first ED access and hospitalization modestly varied.