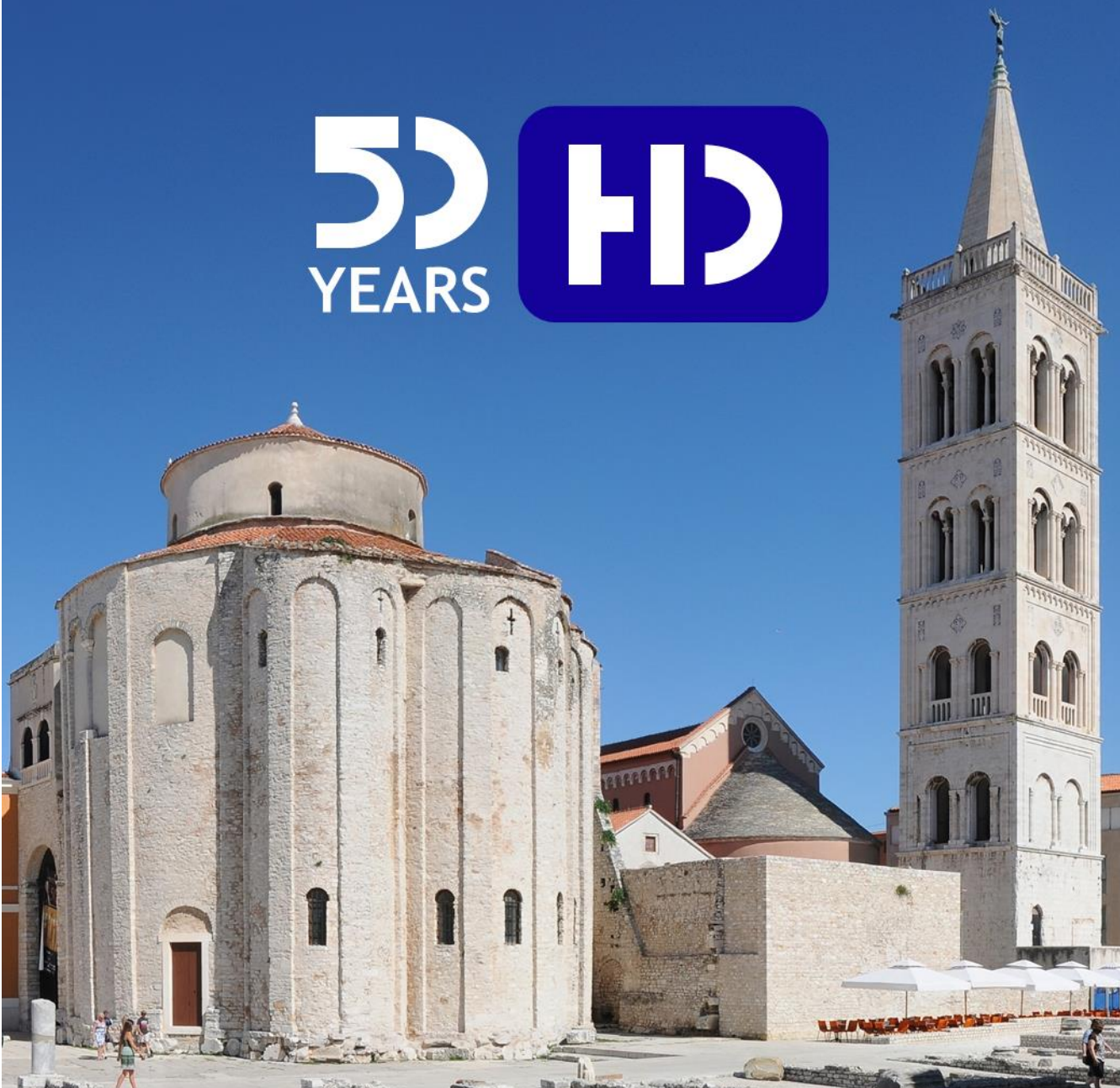
Annual Meeting of the Croatian Immunological Society

Zadar, October 19-20, 2018

50 YEARS HD



Supported by the Foundation of the Croatian Academy of Sciences and Arts

Supported by the Ministry of Science and Education of the Republic of Croatia

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Supported by donations from HEP d.d. & Agmar d.o.o.

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HRVATSKO IMUNOLOŠKO DRUŠTVO
CROATIAN IMMUNOLOGICAL SOCIETY

50 GODINA DRUŠTVA
UTEMELJENO 1968.
FOUNDED 1968
SOCIETY'S 50 YEARS

2018 ANNUAL MEETING OF THE CROATIAN IMMUNOLOGICAL SOCIETY

ZADAR, OCTOBER 19th-20th 2018

ORGANIZED BY

CROATIAN IMMUNOLOGICAL SOCIETY
University of Rijeka Faculty of Medicine
University of Zagreb School of Medicine

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Felix Wensveen, Rijeka

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PROGRAM

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"You never fail until you stop trying."

Albert Einstein

Dear colleagues and guests,

I would like to welcome you at the Croatian Immunological Society meeting that is held, this year, on the special occasion of our 50th anniversary.

Looking back, we can be proud that, for all these years, our immunologists have successfully kept up with the rapidly developing basic and applied immunology at the global level. Immunology in Croatia has been productive, rich, internationally recognized and highly competitive, thereby achieving a prominent place among other scientific disciplines. Different groups in different fields of immunology have contributed significantly to some of the greatest achievements worldwide, pointing out that true scientific values go above and beyond the limitations of time and place.

Immunology has been developing fast, distinguishing itself as a truly interdisciplinary, dynamic and exciting research field that elucidates some of the fundamental biological processes and disease mechanisms. Looking forward, challenges are difficult and expectations are great. To meet these goals, the entire scientific community should continue striving with passion, enthusiasm and respect towards pure research principles and veracity in science.

I wish you an inspiring meeting and spirited future work!

Danka Grčević

President of the Croatian Immunological Society

LECTURES

TWIST1 REGULATES FUNCTION, METABOLISM AND SURVIVAL OF PATHOGENIC TH CELLS DRIVING CHRONIC INFLAMMATION

Andreas Radbruch

Deutsches Rheumaforschungszentrum Berlin and Charité University Medicine, Berlin, Germany

Chronic inflammatory diseases are still a major challenge to biomedical research. Why can we not achieve therapy-free remission in most of the patients? We know that refraction of these diseases to conventional (immunosuppressive) therapies is imprinted in the patient´s immune system: When a patient´s immune system is ablated and regenerated from autologous progenitor cells by the experimental therapy of autologous stem cell transplantation, more than 60% of refractory patients achieve longterm therapy-free remission. This “Immunreset” is eliminating the experienced lymphocytes of the patient, which obviously had driven the inflammation and which had been “roadblocks” to tolerance induction. Upon ablation of these lymphocytes, the patients regenerate a juvenile, tolerant and healthy immune system.

Which types of imprinted “memory” lymphocytes are bystanders, and which ones really drive chronic inflammation, i.e. are pathogenic, and why are these refractory to conventional immunosuppression? We had identified earlier longlived plasma cells secreting pathogenic autoantibodies as one type of pathogenic lymphocytes, and novel target of therapy. Another prime suspects are T helper (Th) lymphocytes, which are refractory to conventional immunosuppressive therapies. Most of these conventional therapeutic strategies have been developed from a basic understanding of acute, protective immune reactions, involving regulatory T (Treg) and B cells, rapid proliferation of the activated lymphocytes and distinct mechanisms regulating apoptosis.

Here we show that in chronic inflammatory diseases of the gut and the joints, Th lymphocytes of the inflamed tissue adapt to the chronic inflammation, and this makes them refractory to conventional therapies. First of all, their proliferation is limited and controlled by microRNA 182, suppressing Foxo1/3a. Then inevitably they develop into Th1 lymphocytes expressing the master transcription factor T-bet, even if they had started out as Th17 cells. They do no longer express IL-2, i.e. do no longer activate Treg. The Th1 inducing cytokine IL-12 and repeated antigenic stimulation upregulate expression of the gene Twist1. Twist1 is an evolutionary conserved E-box binding transcription factor. Haploinsufficiency of Twist1 already leads to cachexia and premature death. Twist1 deficiency of Th cells shows that Twist1 dampens immunopathology of inflammation. It also switches their metabolism to fatty acid oxidation and protects them from reactive oxygen species. Finally, Twist1 controls survival of Th1 cells by inducing microRNA148a, which in turn suppresses expression of the pro-apoptotic protein Bim, and thus protects the cells from apoptosis. Using miR148a-antagomirs in the murine model of transfer colitis, we can selectively eliminate Twist1/miR148a (and PD-1) expressing Th cells. Inflammation is ameliorated as it is by antibody-mediated ablation of all Th cells. Protective immune responses are not affected. This is just one example of the molecular adaption of proinflammatory T lymphocytes to chronic inflammation, demonstrating that these adaptations represent novel and selective targets for a causative treatment of chronic inflammation, but also proving for the first time, that these cells indeed drive chronic inflammation, as has been suspected for a long time.

This work has been supported by the ERC (Advanced Grant “Immemo”) and the DFG (CRCs 633, 650 and TR241).

INTERLEUKIN-6 AND ADAM17 IN INFLAMMATION AND CANCER

Stefan Rose-John

Institute of Biochemistry, University of Kiel, Germany

Cytokines receptors exist in membrane bound and soluble form. The IL-6/soluble IL-6R complex stimulates target cells not stimulated by IL-6 alone, since they do not express the membrane bound IL-6R. We have named this process 'trans-signaling'. The soluble IL-6R is generated via ectodomain shedding by the membrane bound metalloprotease ADAM17. Soluble gp130 is the natural inhibitor of IL-6/soluble IL-6R complex responses. The dimerized recombinant soluble gp130Fc fusion protein is a molecular tool to discriminate between gp130 responses via membrane bound and soluble IL-6R responses.

Colorectal cancer is treated with antibodies blocking epidermal growth factor receptor (EGF-R), but therapeutic success is limited. EGF-R is stimulated by soluble ligands, which are derived from transmembrane precursors by ADAM17-mediated proteolytic cleavage. In mouse intestinal cancer models in the absence of ADAM17, tumorigenesis was almost completely inhibited. Because EGF-R on myeloid cells, but not on intestinal epithelial cells, is required for intestinal cancer and because IL-6 is induced via EGF-R stimulation, we analyzed the role of IL-6 signaling. Tumor formation was equally impaired in IL-$6^{-/-}$ mice and sgp130Fc transgenic mice, in which only trans-signaling via soluble IL-6R is abrogated. Our data reveal the possibility of a novel strategy for treatment of colorectal cancer that could circumvent intrinsic and acquired resistance to EGF-R blockade.

Interestingly, global blockade of IL-6 signaling by neutralizing monoclonal antibodies and selective blockade of IL-6 trans-signaling can lead to different consequences. Inhibition of IL-6 trans-signaling but not global IL-6 blockade was beneficial in several inflammation and cancer models. The extent of inflammation is controlled by trans-signaling via the soluble IL-6R. Therefore, sgp130Fc is a novel therapeutic agent for the treatment of chronic inflammatory diseases and cancer and it underwent phase I clinical trials as an anti-inflammatory in 2013/2014. Phase II clinical trials in patients with autoimmune diseases such as inflammatory bowel disease have recently started in Germany as well as in China, Taiwan and South Korea.

[1] Garbers G, Heink S, Korn T, and Rose-John S (2018) Interleukin-6: Designing specific therapeutics for a complex cytokine. Nat Rev Drug Disc 17: 395-412

[2] Schmidt S et al (2018) ADAM17 is required for EGF-R induced intestinal tumors via IL-6 trans-signaling. J Exp Med 215: 1205-1225

[3] Heink S et al (2017) Trans-presentation of interleukin-6 by dendritic cells is required for priming pathogenic TH17 cells. Nat Immunol 18: 74-85[4] Rose-Rose-John S, Winthrop K, Calabrese L (2017) The role of IL-6 in host defense against infections: Immunobiology and Clinical Implications. Nat Rev Rheumatol 13: 399-409

A NEW ROLE OF AN OLD PLAYER: INTERFERON GAMMA-MEDIATED CROSSTALK BETWEEN THE IMMUNE AND ENDOCRINE SYSTEMS IN VIRAL INFECTIONS

Bojan Polić

University of Rijeka Faculty of Medicine, Rijeka, Croatia

Introduction: Diabetes Mellitus type 2 is a chronic metabolic disorder mostly associated with obesity and characterized by high blood glucose levels and insulin resistance (IR) in peripheral tissues. Prospective clinical studies show that development of DM2 is often associated with an abrupt increase in blood glucose levels after a stable pre-diabetic phase. However, factors which induce this aggravation are largely unknown.

Aim: The aim of this study was to investigate whether and how a viral infection influence glucose homeostasis in pre-diabetic obese subjects.

Materials and Methods: To investigate whether infection impacts glucose homeostasis we setup a small prospective human study in which lean and overweight/obese patients were analyzed for fasting plasma insulin and glucose levels at the time of diagnosis of an acute respiratory infection and 3 months later. In parallel, we used C57BL/6J mice exposed to diet induced obesity (DIO) and subjected to infection with mouse cytomegalovirus (MCMV) at the pre-diabetic phase. To gain insight in the mechanism underlying virus-induced progression of DM2 we neutralized cytokines by mAbs or used appropriate knockout mice for genes encoding cytokines (IFNγ, TNF) or their receptors (IFNγR1, TNFRp55).

Results: We found that respiratory infection increases systemic IR (HOMA-IR) and fasting insulin levels in normal and to more extent in overweight euglycemic patients. Blood glucose levels remained rather normal in both groups. Furthermore, we noticed that three months after infection, systemic IR and insulin levels were decreased in both groups of patients. In mice, we noticed that infection induced strong IR and hyperinsulinemia in both lean and pre-diabetic mice after 7 days. This resulted in normal blood glucose levels in lean mice and in sustained glucose intolerance in pre-diabetic DIO mice. Using genetically modified animals and neutralizing antibodies we identified IFNγ as the major aggravating factor of IR. By conditional deletion of IFNγR1 in tissues important for glucose homeostasis we found that IFNγ specifically targets skeletal muscle but not liver or adipose tissue. Furthermore, we found that IFNγ specifically downregulated expression of the insulin receptor in skeletal muscle, but not in liver. Finally, we identified insulin as a factor that enhances specific antiviral immune response by direct promotion of CD8 T effector functions in vitro and in vivo.

Conclusion: Virus-induced IFNγ causes selective insulin resistance in skeletal muscle and drives hyperinsulinemia to boost anti-viral immune response and derails glycemic control in pre-diabetic obese subjects.

CELLULAR AND MOLECULAR TARGETS OF FAS-DEPENDENT INFLAMMATION AND BONE RESORPTION IN ARTHRITIS

Nataša Kovačić

University of Zagreb School of Medicine, Zagreb, Croatia

Fas/Fas ligand system is a well described regulator of the immune system homeostasis, and involved in the pathogenesis of various immune disorders. This pleiotropic system acts on a wide range of cell types, not only by induction of apoptosis, but also via non-apoptotic signals regulating their proliferation, differentiation and survival. Our group has long been studying the role of this system in the regulation of bone cell apoptosis, differentiation and function, and established that deficiency in either Fas or its ligand has a bone-sparing effect, promoting bone formation over resorption, especially under bone-compromising conditions such as estrogen deficiency and inflammation. We were particularly interested in its role in rheumatoid arthritis (RA) known for its bone-destructive potential, so we analyzed the course of antigen-induced arthritis (AIA, a mouse model of RA) in mice deficient for Fas (Fas –/–), and found less inflammation, alleviated synovial thickening and lack of bone resorption. To evaluate potential mechanisms and downstream molecules involved in reduction of disease activity, we first assessed the cellular composition of the infiltrate in the affected synovia. Amongst the altered populations, the earliest bone and cartilage progenitors (CD45–CD31–TER119–CD200+CD90.1–CD105–) were preserved in Fas –/–mice with ameliorated AIA, in contrast to their reduction in wild-type mice with destructive AIA. Those cells express high levels of Fas and their removal by Fas ligation in arthritis may compromise bone forming regenerative capacity. Proportion of CD200+ cells is also lower in synovia from RA patients in comparison to healthy controls. In addition, Fas inactivation blocked accumulation of myeloid cells (CD11b+Gr1+) in synovial compartment of mice with arthritis. Based on comparison of gene expression, Fas –/– synovial myeloid cells exhibit similar profile to wild-type myeloid cells. Differential expression analysis identified low expression of Midline 1 (Mid 1) gene. Mid 1 encodes a microtubule associated ubiquitine-ligase E3, which enhances the removal of protein phosphatase 2A (PP2A), responsible for suppression of inflammation. Lower expression and function of Mid 1 has been reported in TRAIL deficient mice, pointing to its downstream position to death receptor signaling. Further functional investigation of this gene and its product may reveal new targets for improved therapeutic management of arthritis.

DENDRITIC CELLS AND CELL DEATH DETECTION

Thomas Brocker

Institute for Immunology, Ludwig Maximilians Universität München, Germany

Cell-specific ablation in is an established method to study functions of various cell types in their natural environment. Expression of Diphtheria toxin (DT) itself or DT-receptor in transgenic or knock-in mice is a valuable tool to generate animals lacking a cell type of interest or where this cell type can be ablated upon injection of DT. In the past we have been studying the roles of conventional Dendritic cells (DCs) during infection and immunisations by using different types of DT-mediated ablation approaches. Although cell death has been studied in great detail, it is currently difficult to analyse dead cells in vivo. Therefore we have generated a novel reagent which allows the staining of apoptotic cells in mice. Applied during immune responses we find increase of apoptosis, however, the major change we detect is the substantial coating of immune cells with extracellular vesicles (EVs) in situ. Using AMNIS-flow microscopy combined with a deep learning approach we identify cell types and subsets preferentially coated with EVs and attempt to identify the role and origin of EVs for the immune response during viral infection.

MCMV AFFECTS ENDOSOMAL RECYCLING BY ALTERATION RAB/ARF CASCADE IN INFECTED FIBROBLASTS

Hana Mahmutefendić Lučin

University of Rijeka Faculty of Medicine, Rijeka, Croatia

The final goal of any virus is to exploit the cell for its own replication. With that purpose, cytomegalovirus (CMV) induces remodeling of cellular compartments. The whole process is finalized with the formation of the viral assembly compartment (VAC, viral factory). However, that cellular perturbation results with the disturbance of the normal cellular function, primarily considering its endosomal transport. Therefore, the elucidation of the processes that influence the cellular physiology can help us to understand the mechanisms that virus uses in order to prepare the formation of VAC.

The perturbation of cellular endosomal system takes place very early in the infection (6 hrs p.i.), and is featured with the formation of the EPERC (early-phase endosomal retention compartment). That juxtanuclear (JN) compartment arise as the result of restructuring of early endosomes (EE), endosomal recycling compartment (ERC), and trans-Golgi network (TGN). Normally, the transport through those cellular compartments is very precisely regulated by the Rab/Arf cascades and loops. Rab and Arf proteins belong to the group of the small GTPases that could exists in their GTP (active), or GDP (inactive) form. Therefore, the target of viral activity, when many of cellular molecules that are transported through sorting endosomes (SE) accumulate in EPERC, should be searched for between those molecules. Furthermore, due to the viral interference in normal regulation of endocytic flow, the recycling pathways are also inhibited, and transferrin receptor (TfR), MHC-I molecules (MHC-I), Rae-1, and NBD-sphyngomyelin (NBD-SM) are retained juxtanuclearly. The kinetic studies and mathematic modeling have shown that fast and slow recycling pathways are targeted, but not the ultra-rapid recycling.

Considering retention of specific Rab and Arf molecules in remodeled JN compartment, as well as the amount of their total cellular expression, we have concluded that MCMV targets the Arf6/Rab35 loop and Rab35 effectors (MICAL-L1, Rab13, Rab10, Rab36, Rab8, EHD1), but also, very probably, the Rab11-Rab8/Rab10 activation cascade, and Rab4-Arl1-Arf1/Arf3 cascade. Those MCMV interventions includes: locking of small GTPase into permanently active (GTP), or permanently inactive (GDP) form, loss of GTPase from the cell membrane, etc. The final result is the inhibition of endosomal flow, including also the process of recycling.

Therefore, we can conclude that MCMV interference with Rab/Arf cascade issue with inhibition of cellular recycling. The side effect of that process is also the immunoevasive effect of CMV infection due to downregulation of MHC-I and Rae-1 molecules.

This work was supported in part by the Croatian Science Foundation (Grant IP-2014-9-9564) and by the University of Rijeka (grants: 13.06.1.1.4, 13.06.2.1.55 and 13.06.2.1.56)

TOWARD A GENETIC THEORY OF CHILDHOOD INFECTIOUS DISEASES

Jean-Laurent Casanova

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The hypothesis that inborn errors of immunity underlie infectious diseases is gaining experimental support. However, the apparent modes of inheritance of predisposition or resistance differ considerably between diseases and between studies. A coherent genetic architecture of infectious diseases is lacking. We suggest here that life-threatening infectious diseases in childhood, occurring in the course of primary infection, result mostly from individually rare but collectively diverse single-gene variations of variable clinical penetrance, whereas the genetic component of predisposition to secondary or reactivation infections in adults is more complex. This model is consistent with (i) the high incidence of most infectious diseases in early childhood, followed by a steady decline, (ii) theoretical modeling of the impact of monogenic or polygenic predisposition on the incidence distribution of infectious diseases before reproductive age, (iii) available molecular evidence from both monogenic and complex genetics of infectious diseases in children and adults, (iv) current knowledge of immunity to primary and secondary or latent infections, (v) the state of the art in the clinical genetics of non-infectious pediatric and adult diseases, and (vi) evolutionary data for the genes underlying single-gene and complex disease risk. With the recent advent of new-generation deep resequencing, this model of single-gene variations underlying severe pediatric infectious diseases is experimentally testable.

PERINATAL CYTOMEGALOVIRUS INFECTION DRIVES NK CELL HYPORESPONSIVENESS CHARACTERIZED BY DOWNREGULATION OF T-BOX TRANSCRIPTION FACTOR

Ilija Brizić

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Congenital human cytomegalovirus (HCMV) infection is the most common viral cause of long-term neurodevelopmental sequelae, including mental retardation, microcephaly and sensorineural hearing loss. NK cells have been shown to play an important role in containing cytomegalovirus (CMV) infection and various adaptive features of NK cells in response to CMV infection are recently being increasingly studied. Despite an increasing body of knowledge the involvement of NK-cell mediated immunity in congenital CMV infection is so far largely unknown. Since HCMV is species-specific, we are using a mouse model in which newborn mice are infected with the mouse cytomegalovirus (MCMV). Here we show that perinatal MCMV infection leads to a persistent alteration of transcriptional activity and strongly affects the maturation and function of NK cells. NK cell expression of T-box transcription factor Eomes, critical for NK cell development, was dramatically impaired after infection. The downregulation of Eomes correlated with major changes in the NK cell phenotype, most notably NK cell exhaustion characterized by an impaired NK cell response to different stimuli. This population of NK cells persisted for several months in infected mice indicating that congenital CMV infection shapes NK cell response over long-term period. Altogether, our data indicate that NK cells are strongly affected by the congenital CMV infection.

ORAL PRESENTATIONS

MCMV EXPRESSING HIGH AFFINITY NKG2D LIGAND MULT-1: A MODEL VACCINE FOR CONGENITAL CMV INFECTION

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Introduction: Cytomegaloviruses (CMVs) encode numerous non-essential immunoevasive genes which can be deleted or replaced by any other gene without affecting viral growth or fitness *in vitro*. Deletion of certain viral immunoevasins attenuates the virus *in vivo*, thus enabling the generation of viruses with altered virulence, which can be used as vaccine vectors. We have previously shown that murine CMV (MCMV) expressing RAE-1γ (one of the cellular ligands for the NKG2D receptor) is highly attenuated *in vivo* but retains the capacity to induce strong adaptive immune response to viral and vectored antigens. MULT-1 is a NKG2D ligand with highest affinity for the receptor. We constructed recombinant MCMV expressing high affinity ligand MULT-1 (MULT-1MCMV) and compared it with RAE-1γMCMV and MCMV to further explore vaccine properties of such virus.

Materials and Methods: MULT-1MCMV was constructed by insertion of gene for NKG2D ligand MULT-1 in the place of its viral regulator *m145*. Viral spread *in vivo* was determined by infection of adult and newborn mice and assessment of viral titer in their organs. Capacity to induce CD8 T cell response was determined *in vivo* by evaluating antigen-specific CD8 T cells in infected animals and *in vitro* by CD8 T cell antigen presentation assay. Humoral response was determined by analysis of virus-specific antibodies in the sera of infected mice and offspring of immunized mothers.

Results: We demonstrate that a recombinant MCMV expressing high affinity NKG2D ligand MULT-1 is highly attenuated *in vivo* by NK cells in a NKG2D-dependent. MULT-1MCMV is capable of inducing antigen-specific CD8 T cells and humoral response. In newborn mice, MULT-1MCMV was controlled more rapidly compared to RAE-1γMCMV and failed to reach the brain of infected newborn mice. As a result, newborn mice infected with this virus were devoid of brain inflammation and microglia polarization.

Conclusion: Our study showed that expression of high affinity NKG2D ligand MULT-1 in MCMV mediates its strong immune control while retaining capacity to induce robust cellular and humoral immunity. Efficient virus control and absence of immunopathology of MULT-1MCMV was particularly pronounced upon infection of neonatal mice. These findings demonstrate that CMV-based vaccine vectors expressing high affinity NKG2D ligand are promising vaccine candidates, which could be used in immunologically immature and immunocompromised patients.

EOMES BROADENS THE SCOPE OF CD8 T CELL MEMORY BY INHIBITING APOPTOSIS IN LOW-AFFINITY CELLS

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7,8 These authors contributed equally

Introduction: The effector and memory CD8 T cell pools have an intrinsic difference in the way that they must approach antigen. Effector cells are faced with an actively replicating pathogen and therefore benefit most from selection of efficient high-affinity clones. The memory pool must anticipate that upon re-encounter, a pathogen may have undergone point mutations in immuno-dominant epitopes in response to immunological pressure as it moves through its host population. CD8 T cell memory must therefore contain both high- and low-affinity clones to battle the original pathogen as well as mutants.

Aim: Investigate how cells of high- and low-affinity are selected in to the memory CD8 T cell pool.

Materials and Methods: To investigate how signal strength impacts memory differentiation, we set up an *in vitro* model in which we stimulated OT-1 T cells with SIINFEKL (N4 peptides) or altered peptide ligands (APLs). Using conditional knock-out mice ($Eomes^{fl/fl}$CD4Cre), we showed a crucial role of Eomes for the survival of low-affinity memory cells through induction of Bcl-2. The inducible MXCre system was used to determine the timeframe in which Eomes mediates this pro-survival effect. To confirm this data in polyclonal system we generated mixed BM chimeras, whereas ABT-199 was used to specifically inhibit Bcl-2. To asses if Eomes binds to *Bcl2* promoter region we performed Eomes ChIP-seq on activated OT-1 cells. To gain deeper insight in the molecular mechanism responsible for survival advantage of low-affinity CD8 T cells, we generated transgenic NIH3T3 and HEK cell lines overexpressing Eomes or both Eomes and T-bet. Further on, luciferase assay was performed using plasmids containing a luciferase gene preceded by the promotor of Bcl-2, p100 or CD122 promotor. To confirm that Eomes deficience results with reduced clonal diversity of memory CD8 T cell pool we performed TCR seq of MBMCs infected with LCMV.

Results: We find that low-affinity memory exclusively depends on the transcription factor Eomes in the first days after antigen encounter. Eomes is induced at low activating signal strength and directly drives transcription of the pro-survival protein Bcl-2. At higher signal intensity T-bet is induced which suppresses Bcl-2, generating a survival advantage for low-affinity cells. In contrast, high-affinity cells form memory independent of Eomes, but have a proliferative advantage over low-affinity cells, which compensates for their survival deficit.

Conclusion: We demonstrate on a molecular level how sufficient diversity of the memory pool is established in an environment of affinity-based selection.

THE ROLE OF γδ T CELLS IN DEVELOPMENT OF NAFLD

Sonja Marinović, Ante Benić, Marko Šestan, Inga Kavazović, Felix M. Wensveen and Bojan Polić

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Introduction: Non-alcoholic fatty liver disease (NAFLD) is accumulation of extra fat in liver cells that is not caused by alcohol and it is considered as hepatic manifestation of metabolic syndrome. It represents a wide spectrum of liver disease from simple steatosis to steatohepatitis (NASH) and cirrhosis that leads to end-stage liver failure.

Aim: The aim of this research was to investigate immune mechanisms that are important for induction of inflammatory process and development of NASH. Focus was on γδ T cells and their receptor NKG2D that is known sensor of cellular stress and is able to induce inflammatory response.

Materials and Methods: In our research, we used SSD diet model (40% fat, 20% fructose, 2% cholesterol) that induced all stages of NAFLD within 16 weeks after the start of the diet. We analysed changes in immune cell populations in liver within that time by FACS. By using different *knock-out* mouse strains we investigated the role of γδ T cells and their receptor NKG2D in development of NASH.

Results: Histological examination of hematoxylin and eosin- (HE) and Sirius red-stained liver sections revealed the development of all stages of NAFLD in mice that were on SSD diet. In wild type (WT) mice first few weeks after the start of SSD diet were marked by accumulation of fat in hepatocytes which was followed by an early increase in number of γδ T cells, change of their phenotype that included increase in expression of NKG2D receptor and production of pro-inflammatory IL-17A cytokine.

In comparison to WT control, $TCR\delta^{-/-}$ and $NKG2D^{-/-}$ knock out mice had less pronounced hepatic inflammation and fibrosis when fed with SSD diet. Furthermore, lack of DAP10, a downstream molecule in NKG2D signaling cascade, also resulted in decrease of inflammation and fibrosis.

Conclusion: γδ T cells are immune cell sensors that link diet-induced cellular stress to development of NASH. This process is mediated by NKG2D-induced IL-17A production by γδ T cells.

LOSS OF OPTINEURIN IN MICROGLIA LEADS TO DISBALANCE IN PRO- AND ANTI-INFLAMMATORY FACTORS REGULATED BY IFN-β

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Optineurin is a multifunctional, ubiquitin (Ub)-binding protein that is proposed to regulate numerous cellular processes including inflammatory signalling, autophagy, vesicular trafficking and cell death. All of these processes have been described as pathogenic mechanisms in neurodegenerative diseases. Thus far more than 30 mutations in optineurin have been found in patients with amyotrophic lateral sclerosis (ALS), a progressive neurodegenerative disease, characterized by the loss of upper and lower motor neurons. The majority of characterized ALS mutations in optineurin suggest that it is neuroprotective and causes disease by loss of function.

To assess the role of optineurin in neuroinflammation, which is driven by the brain macrophage termed microglia, we constructed an optineurin insufficiency mouse model with C-terminal truncation of Ub-binding region ($Optn^{470T}$).

In contrast to previous results obtained in cells lines, we found that optineurin is dispensable for NF-κB activation upon TLR4 stimulation with LPS in primary microglia, as measured by IkB degradation, p65 phosphorylation, and TNF production. However, upon stimulation with LPS and poly (I:C), $Optn^{470T}$ microglia had diminished Tank-binding kinase 1 (TBK1) activation, and subsequently phosphorylation of interferon regulatory factor 3 (IRF3) and production of IFN-β, suggesting that optineurin is necessary for optimal activation of this signalling pathway. Fittingly, STAT1 activation was also diminished in $Optn^{470T}$ microglia, leading to a disbalance in expression of several IFN-β-regulated genes, including IRF7, NOS2, CXCL10, IL-10 and CXCL1. However, expression of those pro- and anti-inflammatory factors was rescued upon addition of recombinant IFN-β, indicating that Ub-binding function of optineurin and IFN-β signalling are necessary for optimal inflammatory response of the microglia upon TLR engagement. Similar phenotype of diminished TBK1 signalling activation was also found in $Optn^{470T}$ primary neurons. In spite of these differences in primary cell cultures, short-term systemic LPS application *in vivo* elicited equal microgliosis in WT and $Optn^{470T}$ mice. However, cytokine array analysis of whole brain lysates from $Optn^{470T}$ mice upon LPS challenge showed increased production of granulocyte chemoattractants such as G-CSF, CXCL1 and LIX in comparison to WT mice.

We are currently focusing on understanding the functional repercussions of elevated chemokine production in $Optn^{470T}$ mice. Since no signs of microgliosis were found in the brain and spinal cord of aged $Optn^{470T}$ mice in comparison to WT controls, we hypothesise that additional and/or chronic stimuli are needed for the disease manifestation.

NK CELL-DERIVED IFN-γ CONTRIBUTES TO THE PATHOGENESIS OF MCMV ASSOCIATED ALTERED CEREBELLAR DEVELOPMENT

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Introduction: Human cytomegalovirus (HCMV) is the most common cause of congenital viral infections in humans and may lead to long-term central nervous system (CNS) abnormalities including microcephaly, mental retardation and hearing loss. One of the major obstacles in the study of pathogenesis of HCMV infection is its species-specificity which has restricted the scope of studies of cytomegalovirus infection to animal models. Unlike HCMV, mouse cytomegalovirus (MCMV) cannot pass through the placenta and thus can not cause intrauterine infection. Therefore, we developed a mouse model in which newborn mice are infected intraperitoneally with MCMV. This model recapitulates the major characteristics of CNS infection in human infants of the second trimester of intrauterine development, including the route of viral neuroinvasion and neuropathological findings. Following intraperitoneal inoculation of newborn mice with MCMV, the virus disseminates to the CNS, replicates in the brain parenchyma and causes altered cerebellar development. The initial immune response is dominated by the influx of NK cells, whose appearance coincides with detection of the virus in the CNS. The pathogenesis of the CNS infection has not been completely clarified and may arise as a result of direct damage of MCMV infected neurons or indirectly secondary to inflammation. Despite the important role that NK cells play during MCMV infection in peripheral organs, the role of NK cells upon MCMV infection of the developing CNS remains to be defined.

Aim: The main objective of this study is to determine the role of innate immune cells in brain inflammation and neurodevelopmental abnormalities during congenital CMV infection.

Materials and Methods: To address this issue, we followed the kinetics of NK cell infiltration in the brain and functional state of microglia, and their contribution to neuroinflammation and delay in cerebellar growth during congenital CMV infection.

Results: MCMV infection in brain of newborn mice leads to early infiltration of NK cells, as well as polarization of the microglia towards proinflammatory (M1) phenotype. Here we show that NK cells are an early source of IFN-γ which drives the polarization of microglia towards a proinflammatory phenotype associated with altered cerebellar development in infected newborn mice. Depletion of NK cells or neutralization of IFNγ abolished polarization of microglia and notably, normalized neurodevelopmetnal delay in infected newborn mice.

Conclusion: These results indicate that NK cell derived IFNγ is a major component of the inflammatory response that is associated with altered neurodevelopment that follows perinatal CMV infection.

NKG2D SETS ACTIVATION THRESHOLD FOR NCR1 EARLY IN NK CELL-DEVELOPMENT AND CONTROLS SENSITIVITY OF CANCER IMMUNE-SURVEILLANCE

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Introduction: NKG2D and NCR1 (NKp46) are both activating receptors expressed on all NK cells during NK cell development and have important role in the stress-surveillance. 'Stressed' cells up-regulate NKG2D and/or NCR1 ligands which can engage their receptors and activate NK cells. Previously, our group has shown that NKG2D-deficiency affects NK cell development (Zafirova et al. Immunity 2009). Klrk1$^{-/-}$ mice showed an enhanced NK cell-mediated resistance to MCMV infection, while they kept impaired ability to kill NKG2D-expressing tumor targets.

Aim: Here we investigated molecular mechanism underlaying the NK hyperreactivity and how it influences control of tumors which do not express NKG2D ligands.

Materials and Methods: In our research we used two tumor models: γ irradiation-induced thymoma and B16 melanoma. We also used different functional assays, flow cytometry and various genetically modified mice to investigate roles of specific receptors and signaling molecules.

Results: NKG2D-deficiency results in specific NCR1-medicated NK cell hyperreactivity. The hyperreactivity occurs during the NK cell development and is due to the lack of signaling through NKG2D-DAP12 axis. It is correlated with reduced expression of CD3ζ and Zap70. The hyperreactivity results in better control of the investigated tumors and MCMV infection in Klrk1$^{-/-}$ and DAP12$^{-/-}$ mice.

Conclusion: This research shows for the first time that an activating NK receptor controls activity of another one. Early during NK cell development NKG2D/DAP12 axis sets threshold for NCR1 which leads to NK cell hyperreactivity and better control of MCMV infection and tumors expressing NCR1 ligands.

IL-1R8: A NOVEL CHECKPOINT REGULATING ANTI-TUMOR AND ANTI-VIRAL ACTIVITY OF NK CELLS

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IL-1R8 is an Interleukin-1 receptor family member that acts as a negative regulator of IL-1 family receptor and TLR signaling. Both murine and human NK cells express high levels of IL-1R8 but its functional role in this cell type has not been described so far.

Expression analysis showed that IL-1R8 was acquired during differentiation in human and murine NK cells. IL-1R8 deficiency in the mouse was associated with higher frequency of mature NK cells and enhanced NK cell effector functions. IL-18, which is a key regulator of NK cell activities and can be targeted by IL-1R8, was responsible for this phenotype. Indeed, IL-1R8 regulated IL-18-MyD88 axis during NK cell differentiation and IL-18-dependent activation of mTOR and JNK pathways increased in IL-1R8-deficient NK cells.

To assess the role of IL-1R8 in NK cells in pathology, we used models of MCA-induced lung metastasis, colon cancer-derived liver metastasis and DEN-induced hepatocellular carcinoma. The number and dimension of liver and lung metastasis and the liver disease severity were significantly reduced in *Il1r8*$^{-/-}$ mice. The depletion of NK cells in these models totally abrogated the protection observed in *Il1r8*$^{-/-}$ mice. Finally, we investigated the role of IL-1R8 in NK cell antiviral activity, in a model of MCMV infection. *Il1r8*$^{-/-}$ mice controlled the virus more efficiently in the liver and the protection was associated with enhanced NK cell degranulation and IFN-γ production. The adoptive transfer of *Il1r8*$^{-/-}$ NK cells conferred protection in both metastasis and viral infection models.

IL-1R8 plays a non-redundant role in the regulation of NK cell development and effector functions by tuning IL-18-dependent activities. IL-1R8 therefore emerges as a crucial regulator of NK cell antitumoral and antiviral potential.

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NKG2D MODULATES THE PROINFLAMMATORY FEATURES OF TH1 AND TH17 CELLS AND CONTRIBUTES TO THEIR PATHOGENICITY *IN VIVO*

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The effector functions of T helper (Th) cells can be shaped not only by receiving the T cell receptor and costimulatory signal but also through signals transmitted via cytokine receptors or a myriad of activating receptors. NKG2D is a molecular sensor of stressed cells expressed on different subsets of innate and adaptive lymphocytes. Despite its established role as potent stimulator of the immune system, particularly as an activating receptor on NK cells and costimulatory molecule on $CD8^+$ T cells, NKG2D-driven regulation of $CD4^+$ T helper (Th) cell-mediated immunity remains unclear.

We identified a population of T-bet expressing $NKG2D^+CD4^+$ T cells in spleen and bone marrow of C57BL/6 mice, whereas a significant portion of $NKG2D^+CD4^+$ T cells derived from the small intestine lamina propria expressed RORγt. In line with this, the *de novo* expression of NKG2D could be induced on naïve $CD4^+$ T cells both under Th1 and Th17 polarizing conditions. While NKG2D was only slightly impacting the expression of IFNγ in fully polarized Th1 cells, the *in vitro* expression of NKG2D was associated with $GM\text{-}CSF^+IFN\gamma^+$ Th17 cells. Global gene expression analysis further confirmed enforced expression of type 1 signature genes in Th17 cells by NKG2D and we could show a direct effect of NKG2D triggering in enhancing the production of IFNγ in Th17 cells. By fate mapping of Il17a-expressing cells *in vivo* in a mouse model of antigen-induced arthritis, we could show that under inflammatory conditions, NKG2D highly enriched the population of T-bet-expressing Th17 cells. Indeed, NKG2D was associated with modulated expression of GM-CSF and IFNγ in Th1 and T-bet^+ Th17 cells, which was in line with our *in vitro* data. Most importantly, T cell specific deletion of NKG2D impaired the ability of antigen-specific $CD4^+$ T cells to promote inflammation *in vivo* during antigen-induced arthritis, resulting in significantly reduced knee swelling, tissue immunopathology and disease score in these animals.

Altogether, our results indicate that the triggering of NKG2D by stress-ligands induced during inflammation modulates the effector functions of both Th1 and Th17 cells *in vitro* and *in vivo*. Our data in addition imply that NKG2D might serve as an important target for the amelioration of chronic inflammatory diseases mediated by a mixed Th1 and Th17 response.

A COMPLEX INTERPLAY BETWEEN LY49 RECEPTORS AND CYTOMEGALOVIRUS

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Introduction: Cytomegaloviruses (CMVs) are master immune-evaders. One of their targets are MHC-I molecules which are rapidly downregulated during infection to avoid recognition by CD8 T cells. However, the lack of MHC-I on the surface of infected cells could easily trigger NK cells via "missing-self" killing. Therefore, mouse CMV (MCMV) encodes m04, a protein that binds a small proportion of MHC-I molecules and brings them to the cell surface where they engage inhibitory Ly49 receptors and inhibit NK cell response. Interestingly, while m04 is highly abundant in the cell, only minor fraction rescues MHC-I and leaves the ER.

Aim: To investigate how CMV's ensure proper engagement of inhibitory, and evade engagement by activating Ly49 receptors.

Materials and Methods: We have generated several mAbs and mutant viruses lacking various genes of MCMV, utilize *in vitro* reporter cell assays expressing single Ly49 receptors as well as *in vivo* analyses.

Results: We have identified and characterized 11kDa viral protein MATp1 encoded by the MCMV's most abundant transcript (MAT) that helps in the m04-mediated MHC-I surface rescue and strengthens the interaction between inhibitory Ly49 receptors and their MHC-I ligands. This is relevant *in vivo* since viruses lacking MATp1 are attenuated in mice of various haplotypes in NK-dependent manner. We also show that MATp1 impairs NK cell proliferation and activation through Ly49A–MHC-I axis which leads to inhibition of NK cells and inadequate control of WT MCMV. Furthermore, by analyzing available field isolates of MCMV, we have observed various degrees of differences between them. This prompted us to investigate whether it has been under strong selection pressure by the immune system. Differential sensitivity of various mouse strains to MCMV has been linked to the capacity of NK cells to recognize infected cells via activating Ly49 receptors. We and others have previously shown that activating Ly49D2, Ly49L and Ly49P receptors require MHC I, m04 and additional virally encoded factor for specific recognition of infected cells.

Conclusion: Here we show that the missing factor is MATp1, a protein involved in the recognition by inhibitory Ly49 receptors. Our results indicate that MATp1 originally developed as an immunoevasin targeting inhibitory Ly49 receptors, but has prompted the evolution of activating Ly49 receptors in return. We thus present a novel example by which MCMV evades NK cells and provide additional evidence that MCMV plays a crucial role in the shaping of its host's immune response.

MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF M116 REGION IN MCMV

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Introduction: Human cytomegalovirus (HCMV) is a species-specific herpesvirus that causes severe disease in immunocompromised individuals and congenitally infected infants. Murine cytomegalovirus (MCMV) is biologically similar to HCMV and it serves as a widely used model for studying the mechanism of HCMV infection.

Aim: We have previously identified M116 as one of the most extensively transcribed regions of MCMV genome. Its HCMV homologue, UL116 has been recenty described as an envelope glycoprotein. However, the function of M116 in the pathogenesis of MCMV infection is unknown so our goal is to perform molecular and functional characterization of M116 region.

Materials and Methods: Molecular characterization involved the analysis of the M116 transcripts (Northern blot) and M116 protein (Western blot, flow cytometry and immunofluorescence) from the MCMV infected murine embryonic fibroblasts (MEF). In order to study *in vitro* and *in vivo* properties of M116 a MCMV mutant with deletion in *M116* open reading frame (ΔM116) was constructed and used to determine multistep-growth curves and analyze the function of this virus *in vivo*.

Results: Our molecular characterization revealed at least two 5' co-terminal transcripts in M116 region and at least one glycosylated protein expressed with late kinetics. Growth kinetics of WT and ΔM116 MCMV in primary MEF were comparable, however ΔM116 was attenuated in immortalized macrophages (iBMDM, Raw 264.7), as well as in GM-CSF grown bone marrow derived myeloid cells; BMDCs and BMDMs. Moreover, myeloid cells infected with ΔM116 produced more proinflammatory cytokines IL6 and TNFα. Interestingly, ΔM116 was not severely attenuated *in vivo*, but infection of mice with virus lacking M116 resulted in less inflammation and NK cell activation.

Conclusion: These results are indicating that M116 has an important role in MCMV infection of myeloid cells. Since macrophages and dendritic cells are important for MCMV dissemination and latency, our future work will focus on the mechanism underlying these observations.

PATHOGENESIS OF CYTOMEGALOVIRUS INFECTION IN THE OVARIES

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Introduction: Human cytomegalovirus (HCMV) is a major cause of morbidity resulting of congenital infection and miscarriage during early pregnancy. Although it is well established that human cytomegalovirus can infiltrate the placenta causing congenital disease, little is known about the impact on reproductive organs.

Aim: Analysis of pathogenesis and antiviral response in ovaries of sexually mature female mice upon murine cytomegalovirus (MCMV) infection.

Materials and Methods: The MCMV presence in different ovarian structures (follicles and corpus luteum) was analysed by immunohistochemistry of IE1 viral protein. Antiviral immune response was investigated by using transgenic and knock out mice lacking individual components of the immune cells and signalling pathways.

Results: Our data showed a highly selective MCMV infection in the ovaries among with the strong infection of ovarian stroma and corpora lutea. Yet, no signs of infection in the follicles was observed. Previously, we have shown that follicular resistance to infection depend on the presence of gap junctions and lack of *de novo* vascularization in follicles. Furthermore, a comparative analysis of different imunodeficient mice revealed that follicles of IFNAR KO mice were the most susceptible to infection. Detailed analysis showed significant increase of IFN type I receptor and its downstream factors STAT 1, STAT 4 and OAS 1 in MCMV infected compared to control animals.

Conclusion: These findings implicate that CMV readily infects the ovaries and that follicular antiviral defence is mainly supported by innate immunity with significant contribution from type I interferon.

RETENTION OF ENDOCYTOSED CARGO DURING EARLY PHASE OF MCMV INFECTION

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Introduction: Mouse cytomegalovirus (MCMV) is a member of *Herpesviridiae* family. It is a large DNA virus with highly developed immunoevasive strategy. Upon infecting the host cell it subjects its functions to sole purpose of viral replication and production of virions. Here we show that the MCMV infection leads to extensive changes in the function and the placement of the endosomal compartments that eventually lead to formation of virion assembly compartment.

Aim: The aim of this study was to elucidate the rearrangement of the cellular endosomal system during early phase of MCMV infection and the subsequent consequences regarding intracellular trafficking.

Materials and Methods: Balb 3T3 fibroblasts were infected for 6 hours with recombinant murine cytomegalovirus Δm138-MCMV (ΔMC95.15) that has deleted FcR. Intracellular distribution and expression of endosomal regulatory proteins, endosomal marker proteins and viral proteins was analysed by immunofluorescent confocal microscopy. Cellular kinetic parameters were determined by flow cytometry (using in-house developed software, developed in collaboration with Faculty of Engineering of the University of Rijeka).

Results: Functional assays of the cellular endosomal system showed significant alteration of the endosomal flux during the early phase of MCMV infection. This was demonstrated by following the trafficking of several cargo molecules, such as TfR, MHC-I molecules and NBD-sphingomyelin throughout the endosomal system. Their recycling was delayed and the cargo molecules were predominantly retained in EPERC, the perinuclear endosomal compartment remodelled by MCMV. The kinetic modeling revealed that MCMV infection does not alter the rapid recycling route. However, it showed the inhibition of cargo egress from sorting endosomes (SE) and endosomal recycling compartment (ERC) and the transfer from SE to ERC.

Conclusion: Infection of fibroblasts with MCMV leads to inhibition of egrees from sorting endosomes and endosomal recycling compartment as well as transfer from SE to ERC, but does not affect the rapid recycling route. This leads to retention of endocytosed cargo in SEs and the ERC, which is associated by redistribution of the small GTPases from Rab and Arf families.

This work was supported in part by the Croatian Science Foundation (grant IP-2014-9-9564) and by the University of Rijeka (grants 13.06.1.1.4, 13.06.2.1.55, and 13.06.2.1.56).

VIRALLY INDUCED HYPOGLYCEMIA: GOOD BAD OR BAD BAD?

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Introduction: Hypoglycemia is characterized by a reduction in plasma glucose concentration to a level that may induce conditions dangerous for life. The most common causes of hypoglycemia in non-diabetic patients are alcohol abuse, liver and kidney diseases, tumors, endocrine problems and severe infections.

Aim: Although it is very well documented that severe viral infections can promote hypoglycemia, surprisingly little is known about how viral infection promotes development of hypoglycemia. We hypothesize that virally induced hypoglycemia is a physiological and therefore regulated mechanism that benefits the organism.

Materials and Methods: To address these questions, we employed a Lymphocytic choriomeningitis virus (LCMV) model in which mice were infected intraperitoneally with high doses of LCMV (Amstrong strain) virus. To gain more insights into the mechanism underlying virus-induced development of hypoglycemia we neutralized cytokines by antibodies or used appropriate knockout mice of genes encoding for virus-induced cytokines (IFNγ, TNF, IL-1β) or their receptors (IFNγR1, TNFRp55). To investigate which cell types important for glucose homeostasis are mostly affected by IFNγ we used mouse models for specific ablation of IFNγR1 on adipocytes, hepatocytes and skeletal muscle cells.

Results: We found that mice infected with high doses of LCMV exhibited hypoglycemia. Using genetically modified animals and neutralizing antibodies we identified IFNγ as the major aggravating factor for development of hypoglycemia. By conditional deletion of IFNγR1 in tissues important for glucose homeostasis we found that IFNγ specifically promotes insulin resistance (IR) in skeletal muscle but not in liver or adipose tissue. Skeletal muscle specific IR promotes compensatory hyperinsulinemia, which decreases gluconeogenesis in the liver and promotes development of hypoglycemia. Furthermore, we found that virally induced hypoglycemia increases secretion of type I IFNs which in turns reduced viral replication. This data indicates that hypoglycemia induces the last line of defense during severe viral infections.

Conclusion: Virus-induced IR in skeletal muscle drives hyperinsulinemia to decrease gluconeogenesis in the liver, and to induce antiviral self cell-defending mechanisms to rapidly decrease viral load.

STAT3 SIGNALING IS ESSENTIAL FOR BLADDER CANCER PROGRESSION

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Proper immune response is an important defense mechanism, which deters cancer development and progression. However, chronic inflammation can facilitate tumorigenesis. The role of inflammation in urinary bladder cancer (BC) pathogenesis is poorly understood. It is well documented that one of the main molecular links between inflammation and cancer are interleukin-6 (IL6) and its downstream transcription factor – Stat3.

Here, by using N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)-induced bladder cancer mouse model, we tested the role of IL6 and Stat3 in bladder cancer development and progression. In our experiments we used genetic IL6 deficient mice (IL6 KO) model and mice with urothelial specific conditional deletion of Stat3 as well as chemical inhibitor of Stat3. We employed histopathological, immunohistochemical approaches and gene expression profiling to demonstrate an essential role of IL6 and Stat3 in BC development.

Results show that inhibition of Stat3 activation slows down progression and invasiveness of bladder cancer in BBN-induced mouse model. Interestingly, Stat3 activation in BC occurred largely independently of IL6 signaling, as IL6 deficient mice still developed BC tumors with marked Stat3 activation.

Taken together, our study demonstrates an important role of Stat3 signaling in bladder cancer and creates a rationale to test therapeutic potential of Stat3 inhibitors in patients with bladder cancer.

GLUTAMINE METABOLISM DURING PHENOTYPICAL DIFFERENTIATION OF U937 MYELOMONOCYTIC CELLS

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Introduction: Changes in intracellular metabolic pathways are known to alter functions of immune cells. Our previous study revealed that AICAr, an agonist of AMP-activated kinase, reduced proliferation and increased expression of differentiation markers in U937 myelomonocytic cells.

Aim: Present studies are aimed to test for the role of major metabolic pathways in AICAr-mediated effects.

Materials and Methods: HL-60 and U937 cells were incubated in the presence of AICAr, all-trans-retinoic acid (ATRA) and metformin. The number of viable cells was determined by hemocytometer. Glucose, lactate and ammonia concentrations in supernatants were measured using commercially available kits (Sigma). Autophagy flux was determined by western blot analysis of the levels of LC3B-II and actin in cells treated with or without bafilomycin A. The expression of differentiation markers was analysed by flow cytometry (FACS Calibur). The data are shown as means±S.E.M. and analyzed using Student t-test.

Results: Metformin increased, ATRA decreased and AICAr had no significant effects on glucose consumption and lactate production. However, AICAr significantly increased production of ammonia that was abolished by glutamine deprivation. Both AICAr- and ATRA-mediated increase in autophagy flux did not depend on the presence of glutamine. The lack of glutamine, the addition of glutaminase-1 inhibitor and the presence of pharmacological inhibitor of Jumonji demethylases inhibited expression of differentiation markers in response to AICAr, but had no inhibitory effects on ATRA-mediated differentiation. However, the effects of glutamine deprivation were not prevented by addition of dimethyl-α-ketoglutarate, nucleosides or linoleic acid.

Conclusion: These data suggest that glutamine catabolism is necessary for AICAr mediated phenotypical differentiation of U937 myelomonocytic cells, probably through epigenetic mechanism.

This work has been supported by Croatian Science Foundation under the project IP-2016-06-4581 (to D.V.) and co-financed by the European Union through the European Regional Development Fund, Operational Programme Competitiveness and Cohesion, grant No. KK.01.1.01.0007, CoRE-Neuro.

CROSS-TALK BETWEEN INNATE IMMUNE CELLS, ENZYMES MATRIX METALLOPROTEINASES 2 AND 9, CD68 AND HEAT SHOCK PROTEIN 70 IN THE ETHIOPATHOGENESIS OF ATHEROSCLEROSIS

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Introduction: Atherosclerosis is an autoimmune, inflammatory, metabolic and degenerative disease with genetic and environmental factors in its etiopathogenesis, followed by a high degree of morbidity and mortality leading to disruption of work ability and quality of life. Morphology and plaque material play an important role in initiating the atherosclerotic carotid artery symptoms. The lymphocytic subpopulation plays a key role in the initiation and progression of the disease as well as in the regulation of atherosclerotic lesion, although the precise mechanism of this etiopathogenesis can not yet be established. Three proteins are identified as autoantigens: heat shock proteins (HSPs), oxidized low density lipoprotein (oxLDL) and beta 2 glycoprotein 1 (b2GP1). The aim of this study was to investigate the percentage of CD68 + cells, innate immune cells in patients with atherosclerosis and healthy controls, as well as the expression of MMP-2 and 9 in urine as possible markers for monitoring changes during the atherosclerotic process.

Patients and Methods: Immunological status assays were performed by flow cytometry from peripheral blood cells in patients with severe and mild atherosclerosis. The concentrations of enzymes matrix metalloproteinase-2 and 9 were determined by enzyme immunoassays (ELISA) from urine. Immunohistochemically expression of the heat shock 70 (hsp 70) protein on paraffinic atherosclerotic alterations of carotid arteries were done.

Results: Patients with developed atherosclerosis had statistically significant increases in MMP 2 and 9 enzyme urine levels. In the peripheral blood had statistically significant increases in CD68 + molecule, NKT cells and statistically significant decrease in T regulatory cells. The elevated values of NKT cells correlate with the elevated values of enzymes MMP 2 and 9, and are down regulated with the diminished Tregs values. Our data showed a rise in the expression of heat shock protein 70 in atherosclerotic-modified carotid arteries.

Conclusion: The role of heat shock protein (HSPs) in the atherosclerotic process is insufficiently clarified and controversial. They act as autoantigens and stimulate cellular and humoral immune responses. Since they possess immunoregulatory properties, they may be useful in atherosclerosis as immune modulators of the acquired or inherited immune response. High concentration of MMP 2 and 9 enzymes followed by significant diminished values of Tregs in atherosclerotic patients highlights the importance of regulatory T immunity in atherosclerotic etiopathogenesis and T-regs mediated immune therapy and indicated determination of MMP 2 and 9 as an easy markers for the monitoring of the development of atherosclerosis.

POSTER PRESENTATIONS

SYNOVIAL OSTEOPROGENITOR PHENOTYPE IN PATIENTS WITH RHEUMATOID ARTHRITIS

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Introduction: Rheumatoid arthritis (RA) is a chronic, autoimmune joint inflammation, which results in disability due to irreversible joint destruction. Current treatments can slow the progression of the disease, but are still ineffective in a number of individuals and mainly target inflammation. Since there is increasing evidence on the ability of mesenchymal cells to promote regeneration and suppress inflammation, we focused on the phenotype of non-hematopoietic progenitor populations in synovial tissue of patients with arthritis.

Materials and Methods: We analyzed cellular composition of synovial tissue from 9 RA patients undergoing surgery, and 6 control patients undergoing arthroscopic treatment. Cells were released by collagenase digestion and analyzed by flow cytometry after labeling with two panels: 1. CD3-FITC, CD14-PE, 7-AAD, CD11b-PECy7, CD235a-APC, CD19-APCeF780, and 2. CD140a-PE, 7-AAD, CD105-PECy7, CD45/CD31/CD235a-APC and CD200-APCeF780.

Results: Synovial infiltrate in RA had higher proportions of CD3+ and CD19+ cells, and similar variable proportions of CD11b+ and CD14+ in comparison to control samples. Amongst non-hematopoietic (CD45-CD31-CD235a-) cells, proportion of CD105+ cells was significantly increased in RA patients, whereas proportion of CD200+ cells was similar to controls. Amongst CD200+ cells, CD200+CD105+ population was more abundant, while CD200+CD105- cells were slightly less abundant in RA samples in comparison to healthy controls.

Conclusion: There are significant differences in the composition of synovial non-hematopoietic compartment between RA patients and healthy synovia. According to experimental studies, CD200+ cells are considered as earliest osteoprogenitors, and also implicated in regulation of myeloid cell accumulation and activity. Loss of these cells might favor inflammation and arthritis progression.

This work was supported by the Croatian Science Foundation project number 7406.

OSTEOCLAST PROGENITORS EXPRESSING CCR2 ARE EXPANDED IN COLLAGEN-INDUCED ARTHRITIS AND MAY BE INVOLVED IN BONE RESORPTION INTENSITY

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Introduction: Osteoclast progenitors (OCP) originate from the myeloid lineage and mature to osteoclasts, specialized bone resorbing cells. Osteoclast-mediated bone and joint destruction is characteristic for rheumatoid arthritis (RA). OCPs are normally present in bone marrow and among circulatory monocytes. Under inflammatory conditions in RA OCP subpopulations possibly go through various changes and get attracted to inflamed sites by yet unknown mechanisms and chemotactic signals.

Aim: We investigated frequencies of different OCP subpopulations in periarticular bone marrow of affected joints (PBM) and their osteoclastogenic potential in mice with collagen-induced arthritis (CIA), a mouse RA model.

Materials and Methods: After receiving Ethics approval, C57BL/6 and DBA mice were immunized with chicken type II collagen to induce CIA, which was confirmed by micro-CT, histology, and serum CTX levels. Distal tibia bone marrow (PBM) cells were immunophenotyped for hematopoietic markers and chemokine receptor expression. Sorted OCP subsets were assessed for TRAP+ osteoclast number by culturing with M-CSF/RANKL and proliferative response by CFSE. For *in vitro* migration assay, sorted OCPs were seeded into transwell inserts with chemotactic gradient. Mice developing CIA (day 15-23 after immunization) were treated *in vivo* with methotrexate (2mg/kg) and CCR2 receptor antagonist (CRA) (4mg/kg) to assess effects on OCP frequency.

Results: Arthritic mice showed histological presence of BM osteitis and bone destruction assessed by micro-CT and CTX levels. Frequency of CD45+B220-CD3-NK1.1-Ly6G-CD11b−/loCD115+ OCPs was significantly increased in CIA (54% vs. 26% in control), with specific expansion of the CCR2+ subset. Regarding the CCR2 expression level, the CCR2lo subset underwent more divisions and generated multinucleated TRAP+ osteoclasts more efficiently, whereas the CCR2hi subset generated osteoclasts only when cultured at high density. OCPs from CIA mice demonstrated significantly enhanced migration toward CCL2 gradient. Our preliminary results indicate that a combined methotrexate/CRA treatment may affect the frequency of OCPs.

Conclusion: OCP subset expressing CCR2 may contribute to increased homing of OCPs to bone surfaces of inflamed joints and enhanced bone resorption in arthritis. Therefore, inhibition of CCL2/CCR2 signaling presents a new therapeutic approach to reduce osteoclast activity.

SUCCESSFUL TREATMENT WITH ETANERCEPT OF AN ADOLESCENT WITH SUSPECTED TUMOUR NECROSIS FACTOR RECEPTOR-ASSOCIATED PERIODIC SYNDROME ACCOMPANIED WITH INCREASED SERUM IgD

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Introduction: Tumor necrosis factor receptor-associated periodic syndrome (TRAPS) is an autosomal dominant autoinflammatory disorder (AID) caused by mutations in the tumour necrosis factor (TNF) receptor superfamily 1A (TNFRSF1A) gene encoding the 55-kDa receptor for TNF-alpha. Structural mutations in TNFRSF1A tend to have a penetrance higher than 90%, except for P46L and R92Q variants. The diagnosis of TRAPS in patients with these variants relies on clinical judgment or proposed criteria.

Methods: We evaluated the clinical presentation, laboratory findings, genetic analysis and treatment in a 12 year old girl with suspected TRAPS and increased serum IgD.

Results: Our patient had two-year history of recurrent fever attacks lasting from 1 to 3 weeks, myalgia, arthralgia, conjunctivitis, periorbital edema and abdominal pain. Laboratory findings showed increased neutrophils and acute phase reactants during fever attacks, as well as normal serum IgA and increased IgD (max 1350 mg/L) both during attacks and under basal condition. Analysis of the TNFRSF1A gene identified a P46L variant, while no mutation in MVK was found. Short-term corticosteroids failed to control the disease and a therapy with etanercept was initiated, resulting in resolution of symptoms and normalization of inflammatory markers while levels of IgD remained high during follow-up of 12 months.

Conclusion: Interpretation of the P46L sequence variant in patients with suspected TRAPS can be difficult. Since no mutation in MVK gene was found in our patient, this finding further highlights the fact that even in the context of hereditary recurrent inflammatory syndromes, an increase in the serum IgD level is not specific for HIDS.

JUVENILE IDIOPATHIC ARTHRITIS-LIKE POLYARTHRITIS AS A PRESENTATION OF PRIMARY IMMUNODEFICIENCIES

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Introduction: Chronic arthritis in a child younger than 16 years of age, following exclusion of infectious causes, should raise the suspicion of juvenile idiopathic arthritis (JIA), a most common rheumatologic disease in children with complex multifactorial polygenic etiology. Ample evidence shows that a substantial proportion of patients with primary immunodeficiencies (PID) develop JIA-like arthritis as a clinical manifestation of PID.

Methods: The aim of our study was to investigate the prevalence and clinical presentation of JIA in patients who had arthritis as a primary manifestation of PID and those who developed arthritis after PID has been diagnosed. A cohort of 310 patients with a presentation of arthritis that fulfills the International League of Associations for Rheumatology (ILAR) classification of JIA evaluated at our hospital was analyzed. Patient's charts were reviewed for the following immunological investigations performed as part of the diagnosis process: IgG, IgA, IgM, IgE, IgG subclasses, C3, C4, ANA, rheumatoid factor (RF) and HLA-typing. Additional screening and specialized laboratory tests were performed in patients who presented with the recently revised warning signals for primary immunodeficiency.

Results: Seven patients with PID that fulfilled ILAR's criteria for polyarthritis (one for each) were identified. Three patients had selective IgA deficiency (sIgAD), while one patient with the following PIDs in each group was found: ataxia teleangiectasia (AT), autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), autoimmune lymphoproliferative syndrome (ALPS) and familial Mediterranean fever (FMF). Majority of patients presented as RF-negative polyarthritis. Four patients had no diagnosis of PID before arthritis onset.

Conclusion: These findings further emphasize the complex background of many pathophysiologic pathways and clinical presentations of monogenic PIDs that result in autoimmune diseases such as JIA. Our results suggest that JIA-like arthritis should be considered as one of the warning signs for PID.

THE LONG PENTRAXIN 3 PLAYS A ROLE IN BONE TURNOVER AND REPAIR

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Introduction: Pentraxin 3 (PTX3) is an inflammatory mediator acting as a fluid-phase pattern recognition molecule and playing an essential role in innate immunity and matrix remodeling. Inflammatory mediators also contribute to skeletal homeostasis, operating at multiple levels in physiological and pathological conditions.

Aim: The aim of this study is to investigate the role of the inflammatory mediator PTX3 during physiological bone remodeling and fracture healing.

Materials and Methods: Ptx3−/− (knockout) mice were used on a 129/SvPas or C57BL/6J inbred (backcrossed for 11 generations) genetic background (Garlanda et al, Nature 2002). Micro-computed tomography (μCT) and bone histomorphometry were performed to asses bone metabolism. Closed transverse diaphyseal fractures of tibias were created using the method of Bonnarens and Einhorn. Isolated cell suspensions were stained for multiple hematopoietic and stromal markers, and analysed by flow-cytometry. Osteoclasts and osteoblasts were differentiated *in vitro* by the addition of RANKL/M-CSF or ascorbic acid/β-glycerophosphate. In some experiments, either full-length PTX3, its N- or C-terminal domain (50 nM for all), or/and FGF2 (0.25 nM and 0.5 nM) were added to osteoblastogenic cultures.

Results: Ptx3-/- female and male mice had lower femoral trabecular bone volume than their wild-type littermates (BV/TV by μCT: 3.50 ± 1.31 vs 6.09 ± 1.17 for females, $p < 0.0001$; BV/TV 9.06 ± 1.89 vs 10.47 ± 1.97 for males, $p = 0.0435$). In addition, μCT revealed lower trabecular bone volume in second lumbar vertebra of ptx3-/- mice. PTX3 was increasingly expressed during osteoblast maturation *in vitro* and was able to reverse the negative effect of FGF2 on osteoblast differentiation. This effect was specific for the N-terminal domain of PTX3 that contains the FGF2-binding site. By using the closed transversal tibial fracture model, we found that ptx3-/- female mice formed significantly less mineralized callus during the anabolic phase following fracture injury compared to ptx3+/+ mice (BV/TV 17.05 ± 4.59 vs 20.47 ± 3.32, $p = 0.0195$). Non-hematopoietic periosteal cells highly upregulated PTX3 expression during the initial phase of fracture healing, particularly CD51+ and αSma+ osteoprogenitor subsets, and callus tissue exhibited concomitant expression of PTX3 and FGF2 around the fracture site.

Conclusion: We concluded that PTX3 supports maintenance of the bone mass possibly by inhibiting FGF2 and its negative impact on bone formation. Moreover, PTX3 enables timely occurring sequence of callus mineralization after bone fracture injury. These results indicate that PTX3 plays an important role in bone homeostasis and in proper matrix mineralization during fracture repair.

THE EXPRESSION OF GP96 IN VARIOUS MOLECULAR TYPES OF BREAST CANCER

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Introduction: Breast carcinoma is the most common malignant disease in the female population and one of the leading causes of death among women worldwide. At present, various breast tumor classification systems are utilized, more commonly adopted are the TNM classification and immunohistochemistry classification. Hormone receptors-positive carcinomas are the less malignant and have a better clinical prognosis, whereas HER-2 positive and triple negative carcinomas are more aggressive with greater malignancy and a poorer prognosis. Glycoprotein "gp96" is the main heat shock protein within the HSP90 group and is considered a negative prognostic and predictive factor.

Materials and Methods: We conduct an observational retrospective study, processing and analysing patient tissue samples from the archives of the Department of Pathology and Pathological Anatomy (University Hospital Rijeka). The onset of analysis began, with previously immunophenotyped tissue biopsies of the various groups of carcinomas (Luminal A, Luminal B HER2-, Luminal B HER2+, HER2+, triple negative), using "tissue microarray" (TMA) and later, immunohistochemistry staining of these samples was be performed (using gp96 monoclonal antibodies). After that the number of gp96 positive cells was quantified microscopically and the statistical analysis was done using student-t test. These results were compared with clinical data associated with the course and outcome of the disease (age, recurrence of local disease, metastasis and lethal outcome) using hospital's computer information system.

Results: We have found the lowest gp96 expression in the Luminal A and Luminal B HER2- breast cancers group, the highest gp96 expression was in HER2+ and tripple negative breast cancers group among the molecular subtypes. The statistical analysis of the results have shown the statistical significance difference among the gp96 expression in the HER-2 positive and triple negative breast cancers compared to hormonal positive breast cancers (luminal A group) – $p<0,05$. The women in group of HER-2 positive and triple negative breast cancers had poor prognosis (short time to metastasis and recurrence of the disease) when compared to group of luminal A breast cancers that had better clinical outcome.

Conclusion: The goal of this study was to show the difference in the expression of glycoprotein "gp96" among the different groups of breast carcinomas and to incorporate the obtained results with the clinical course and disease outcome in order to gain a better understanding of the clinical and pathological behaviour of various breast cancer immunophenotypes.

This work has been fully supported by the University of Rijeka under the project number 18.07.2.1.02

ONGOING EVOLUTION OF A NOVEL HUMAN METAPNEUMOVIRUS SUBCLUSTER IN CROATIA

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Introduction: Human metapneumovirus (HMPV) is a ubiquitous pathogen of the *Pneumoviridae* family which causes serious respiratory illness in infants, young children, the elderly, and immunocompromised individuals.

Previous reports have shown that the viral attachment glycoprotein (G), one of the major envelope glycoproteins, modulates innate and adaptive immune responses, leading to incomplete immunity and promoting reinfection. Our recent study on genomics of HMPV circulating in Croatia has shown occurrence of a novel virus subcluster A2c within group A genotype. The aim of this study was genetic characterisation of highly diverse G gene of this recently emerged subclaster.

Materials and Methods: This study included 49 nasopharyngeal specimens obtained from hospitalized patients with respiratory infections in Croatia (in 2017) shown to be positive for HMPV in direct-fluorescence assay. All samples were genotyped by amplifying and sequencing a 473 nucleotide long region of fusion protein. Further molecular analyses were conducted for strains belonging to subcluster A2c based on the complete sequence of G gene.

Results and conclusion: In 2017, A2c subcluster has completely replaced all other group A subclusters and accounted for 75% of infections ih hospitalized patients. To gain more insight into evolution of G gene, we included all A2c strains from previous seasons (2011-2016) in phylodynamic analysis and showed substationally higher evolutionary rate of G gene than previously observed. Further surveillance will clarify if this local rate will follow the observed global trend in the near future.

Sequence analysis of complete G gene also showed that this gene is rapidly evolving by gaining two large duplications, either 111 (in 5 of 19 strains) or 180 (in 11 of 19 strains) nucleotides long leading to 37 or 60 amino acid longer G proteins, respectively. Such duplications were recently found within HMPV strains circulating in Spain and Japan. No potential acceptor sites for N-linked sugars have been added by these duplications. Due to heavily glycosylated nature of G gene, estimation of O-glycosylation pattern was not straightforward, but strains containing duplications potentially have more acceptor sites for O-linked glycans.

Additionally, amino acid changes were observed between the first and the second copy of the duplicated region further demonstrating ongoing virus evolution in an extremely narrow time frame. Changes in this region may account for the ability of this virus to infect/reinfect hosts either by enhanced attachment specificities or evasion of immune recognition by the host.

INTERACTION OF MI-I/II WITH MEGALIN ACCELERATES AKT-1 PHOSPHORILATION IN CORTICAL NEURONS AND CONTRIBUTES TO NEUROGENESIS IN CUPRIZONE MODEL OF DE- AND REMYELINATION

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Introduction: Copper chelator cuprizone (CPZ) is neurotoxicant, which selectively disrupts oligodendroglial respiratory chain, leading to oxidative stress and subsequent apoptosis. Demyelination is, however, followed by spontaneous remyelination owing to the activation of intrinsic CNS repair mechanisms. To the later contribute also cysteine rich metallothioneins (MT), which through free radical scavenging and intracellular Zn/Cu regulation provide cytoprotection. Besides, it has been postulated that secreted MT-I/II might be bind on surface receptors belonging to the family of low-density lipoprotein receptor related proteins (LRP), such as LRP-2/megalin and LRP-1, which in turn activate the signal transduction pathways that support neurite outgrowth and survival.

Aim: The goal of this study was to visualize MT/ megalin interaction in the brain tissue of mice affected by CPZ and to determine if this binding leads to the activation of serine/threonine-protein kinase-AKT-1/Protein kinase B signaling cascade.

Materials and Methods: Experiments were performed in female C57BL/6 mice fed with 0.25% CPZ during 5 weeks. MT/megalin co-localization and interactions were examined by double immunofluorescence and proximity ligation assay (PLA), which enables *in situ* recognation of two potentially interacting proteins, respectively. The post-translational modifications in target cells were evaluated by the presence of phosphorylated AKT-1 (pAKT-1; phospho threonine 308).

Results: CPZ-induced demyelination was followed by high astrogliosis and enhanced expression of MTs and megalin in white and gray matter of the brain. PLA clearly showed that MT-I/II interacted with megalin in cortical tissue and in hippocampal subgranular zone of dentate gyrus. Moreover, in most of megalin expressing cortical NeuN+ neurons nuclear expression of pAKT-1 was found.

Conclusion: The data imply that astrocyte-derived MT-I/II modulates cell signaling and neuronal repair through direct contact with megalin and suggest that internalization of MT-I/II and accelerated phosphorylation of AKT-1 contribute to activation of signal transduction pathways that protect cortical neurons and neuronal progenitors against toxic effects of CPZ.

Funded by the University of Rijeka, Croatia (projects 13.06.1.1.16 and 13.06.2.2.58)

HYPERINSULINEMIA IMPAIRS MEMORY CD8 T CELL FORMATION

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Introduction: Diabetes mellitus type 2 (DM2) is associated with hyperglycemia and hyperinsulinemia, but also with reduced immune cell function. Recently, we found that during infection, insulin stimulates the antiviral CD8 T cell response.

Aim: We hypothesized that in DM2, chronic hyperinsulinemia affects CD8 T cell responses.

Materials and Methods: OT-1 T cells were stimulated *in vitro* with ovalbumin peptides and anti-CD28 in presence or absence of insulin. Cells were analyzed by flow cytometry and Seahorse extracellular flux analyser. *In vivo*, WT mice injected with OT-1 T cells were injected with Tresiba basal insulin and infected with mCMV-Ova. Obesity was induced by 12-week feeding with high-fat diet. Animals were injected with OT-1 cells and infected with mCMV-Ova. Recall responses were analyzed by re-infecting animals with LCMV-Ova or by injecting B16-Ova and analysis of metastases in lung.

Results: We find that acute exposure to insulin promotes induction of glucose transporters and enhanced glucose uptake. Extracellular flux analysis showed that insulin stimulation increases glycolytic metabolism in activated CD8 T cells. In addition, insulin promotes effector CD8 T cell differentiation and cytokine production. In obese mice, the hyper-insulinemic state resulted in only a slight increase of the effector response following infection with mCMV. However, we observed that memory cell formation was strongly impaired in these mice. Indeed, obese mice showed a reduction in their capacity to mount a secondary effector CD8 T cell response upon re-infection. Also, after priming with mCMV-Ova obese mice had impaired capacity to lower the number of metastases following B16-ova melanoma injection. Importantly, obesity was associated with reduced insulin responsiveness of CD8 T cells.

Conclusion: Thus, whereas acute insulin exposure promotes the effector CD8 T cell response, in obese mice this results in impaired memory formation and a reduced ability to mount recall responses

INSIGHT IN DIFFERENCES IN VENOM AND ANTIVENOM PHARMOKINETICS IN SERA OF *V. AMMODYTES* BITTEN PATIENTS TREATED BY CURRENTLY AVAILABLE THERAPIES

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Due to current shortage in Zagreb antivenom availability, in Slovenia *V. ammodytes* venomous bites have recently been treated with antivenoms of French and UK producers. In Croatia, for therapeutic purposes the remaining doses of Zagreb antivenom have still been used. Composition differences between these three antivenoms exist. First, they differ slightly in specificity. Zagreb antivenom has been raised against *V. a. ammodytes* venom, but is clinically effective and used against all European venomous snakes (*V. ammodytes, V. berus, V. aspis, V. lebetina* and *V. xanthina*). Viperfav from the French producer has been raised against the mixture of V. aspis, V. berus and *V. ammodytes* venoms, but was mostly used against *V. aspis* and *V. berus*. ViperaTAb from the UK has been raised against *V. berus* venom solely, and used so far only to treat envenomations caused by *V. berus*. Second, they differ in type of the active drug component. Namely, Zagreb antivenom and Viperfav are $F(ab')_2$-based preparations, while ViperaTAb is formulated of Fab fragments. And third, investigated antivenoms have differently prescribed administration routes - intravenous for ViperaTAb and Viperfav or intramuscular for Zagreb antivenom.

Since ViperaTAb's and Viperfav's therapeutical suitability for use against *V. ammodytes* venom-induced toxicity lately has been reported as poorer in comparison to that of Zagreb antivenom, we aimed for elucidation of their distinct clinical efficiency. In serum samples of *V. ammodytes*-envenomed and treated patients, collected during the few days long hospitalization, concentrations of venom, neurotoxic ammodytoxins and $F(ab')_2$ or Fab fragments were determined. Differences in pharmokinetic profiles of investigated antivenoms were revealed. Antivenom level in circulation was highly dependent on the active principle type and route of administration, significantly affecting venom systemic clearance and consequently, clinical status of the patient. Overall, the treatment of patients with Zagreb antivenom did not required additional doses, in contrast to particularly ViperaTAb treatment. This effect might be due to higher specificity of Zagreb antivenom for *V. ammodytes* venom, but also due to differences in administration route and antivenom pharmacokinetics.

ANTI-LEUKEMIC EFFECTS OF AICAR ON MONONUCLEAR BONE MARROW CELLS ISOLATED FROM PATIENTS WITH *DE NOVO* ACUTE MYELOID LEUKEMIA

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Introduction: All-*trans*-retinoic acid (ATRA)-induced differentiation of acute promyelocytic leukemia along the granulocytic lineage provides the most successful example of differentiation therapy of human cancer. Several recent studies suggest that changes in metabolism are involved in differentiation of immune cells. Our previous studies show that 5-aminoimidazole-4-carboxamide ribonucleoside (AICAr), an AMP-kinase (AMPK) activator, increases the expression of differentiation markers in monocytic U937 cell line.

Aim: to test for the possible antiproliferative and differentiative effects of AICAr on mononuclear cells isolated from bone marrow of patients suffering from *de novo* acute myeloid leukemia (AML).

Materials and Methods: HL-60 and U937 cells were obtained from ECACC. Mononuclear cells from bone marrow of AML patients were obtained by density gradient separation and seeded in liquid medium containing 50 ng/mL interleukin-3, interleukin-6, stem cell factor and FLT3-ligand. Cells were collected after obtaining patients' written informed consent in accordance with the Declaration of Helsinki and approval obtained by University Hospital Centre Zagreb Institutional ethic committee. AICAr, ATRA and metformin were obtained from Sigma. Cell viability was determined by MTT assay and the expression of differentiation markers was analyzed by flow cytometry (FACS Calibur). The data are shown as means±S.E.M. and analyzed by Student t-test.

Results: AICAr dose dependently reduces the number of viable myeloblastic HL-60 and monocytic U937 cells. In HL-60 cell line AICAr enhances ATRA-mediated increase in CD11b. In U937 cells, AICAr alone enhances the expression of CD64. *In vitro* profiling of the sensitivity of primary AML samples was performed on mononuclear cells that were isolated by gradient centrifugation from bone marrow of 5 patients with *de novo* AML. Cells were collected after overnight adherence to plastic and seeded in liquid medium in presence of cytokines. MTT assays performed after 96h revealed a significant decrease in viability after treatment with AICAR. An aliquot of these cells was analyzed for the expression of CD11b, CD34, CD45 and CD64. In some patient samples, AICAr showed an increase in CD64 differentiation marker on blast cells, while the expression of hematopoetic stem cell marker CD34 in $CD34^+$ AML cases showed great variability which is in line with the biological heterogeneity of AML.

Conclusion: Preliminary data suggest that AICAr exhibits profound antiproliferative effects on primary AML samples *in vitro*.

This work has been supported by Croatian Science Foundation under the project IP-2016-06-4581 (to D.V.) and co-financed by the European Union through the European Regional Development Fund, Operational Programme Competitiveness and Cohesion, grant No. KK.01.1.01.0007, CoRE-Neuro.

WEAKENED GRANULYSIN-MEDIATED TROPHOBLAST APOPTOSIS IN BLIGHTED OVUM AND MISSED ABORTION

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Introduction: Granulysin is a cytotoxic and pro-inflammatory molecule abundantly present in cytoplasmic granules of decidual NK and T lymphocytes during normal early pregnancy. It is responsible for trophoblast killing in spontaneous abortions. Blighted ovum (BO) and missed abortion (MA) are early pathological pregnancies with hindered development of the embryoblast or a dead embryo, respectively.

Aim: to analyze whether granulysin-mediated apoptotic mechanism is responsible for delayed termination of pregnancy.

Materials and Methods: Granulysin was analyzed by immunohistology of paraffin embedded decidual tissue sections from normal pregnancy (NP), BO and MA and quantified using the Alphelys Spot Browser 2 integrated system (Alphelys, Plaisir, France).

Tissue distribution and relationship of granulysin and cytokeratin expressing trophoblast cells were analyzed by double immunofluorescence. mRNA for granulysin was determined and compared by RT-PCR in freshly isolated decidual mononuclear cells (DMCs) obtained by enzymatic digestion and gradient density centrifugation from NP, BO and MA. The significance among groups was analyzed using the Kruskal-Wallis and Mann–Whitney U non-parametric tests.

Results: Granulysin mRNA was down-regulated in BO and up-regulated in MA, for approximately four times, when compared to NP. Granulysin positive cells were randomly scattered in decidual stroma of NP, BO and MA, and did not differ in the number and staining intensity. Granulysin in NP decidua show dominantly cytoplasmic, while in BO and MA nuclear labelling. In NP granulysin labelled intensively in the nuclei of extra-villous trophoblast cells and cells lining the glands while nuclei of trophoblast cells in BO and MA fluoresce weakly as quantified by H score.

Conclusion: Lower granulysin expression in the nuclei of trophoblast cells in BO and MA when compared to NP, suggests that weakened granulysin-mediated trophoblast apoptosis is responsible for retention of fertilization products in early pathological pregnancy.

Acknowledgements: The investigation was financed by the University of Rijeka Science support No. 13.06.1.1.06 to Daniel Rukavina.

TRANSCRIPTIONAL PROFILING OF SYNOVIAL MYELOID CELLS TO DETERMINE MOLECULAR MEDIATORS OF BONE RESORPTION IN ANTIGEN – INDUCED ARTHRITIS

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Introduction: Rheumatoid arthritis (RA) is a chronic systemic autoimmune arthropathy, which often causes permanent joint damage due to local cartilage and bone resorption. Despite progress toward achieving disease remission current therapeutics are still unable to reverse local bone resorption. Using antigen-induced arthritis (AIA), a murine model of RA, we found that Fas gene deletion (Fas-/-) results in an ameliorated form of arthritis, characterized by abscence of subchondral bone destruction, and marked by a lower frequency of myeloid (CD11b+Gr1+) cells in the synovial compartment.

Aim: By analyzing differentially expressed genes in sorted myeloid populations from wild-type (WT) and Fas -/- mice with AIA we aim to identify myeloid-specific molecular mediators driving bone-resorption in AIA.

Materials and Methods: AIA was induced by intra-articular injection of methylated bovine serum albumin to previously immunized WT and Fas -/- mice. µCT was used to quantitatively assess subchondral bone volume. Synovial tissue was digested by collagenase and released cells were labeled with anti-mouse CD45-FITC, CD11b-PE, Gr1-PECy7, B220/CD3/NK1.1/CD31/TER119-APC, and CD51-APCeF780. CD11b+Gr-1+ population was sorted using BD FACSAria II, and RNA isolated from sorted cells was hybridized to Affymetrix ST 2.0 arrays. Analysis of gene expression data was preformed using Bioconductor and ToppGene. qRT-PCR was used to confirm expression differences in sorted populations and total joint tissue extracts.

Results: Synovial CD11b+Gr1+ population is transcriptionally similar in Fas -/- and WT mice with AIA. Hierarchical clustering based on gene expression data separated two groups of synovial myeloid cells. Each cell group was predominantly represented by either Fas -/- or WT samples, where WT-dominant cluster revealed up-regulation of genes related to cell cycle progression and mitotic activity. Differential gene expression analysis revealed down-regulated *Mid1* and *Erdr1* genes in Fas -/- synovial myeloid cells. According to PCR validation performed on bulk joint tissue, *Mid1* is clearly up-regulated in AIA in comparison to non-immunized WT mice and is upregulated only after arthritis induction in immunized mice.

Conclusion: Resorptive AIA is characterized by increased frequency of synovial myeloid cells, which express more *Mid1* and *Erdr1*, and less *Thbs1* gene, in comparison to non-resorptive arthritis in Fas –/– mice. The inflammatory response in resorptive AIA is marked by a higher myeloid proliferation potential. *Mid1* gene is a potential novel mediator for inflammation-mediated joint destruction in arthritis since it is clearly upregulated by induction of arthritis. Increased expression of *Mid1* has already been reported in an allergic airway inflammation, and it is dependent on death receptor TRAIL.

LIPOPOLYSACCHARIDE-INDUCED ACUTE INFLAMMATION PROTECTS MICE FROM FAS-MEDIATED APOPTOSIS THROUGH STAT3 DEPENDENT PATHWAY

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Introduction: Apoptosis is increasingly recognized as one of the crucial mechanisms in liver injury. It is involved in both, chronic liver diseases and acute liver injuries. However, the nature of inflammation-apoptosis crosstalk in liver is still under research. As we had previously found that acute inflammation induced by lipopolysaccharide (LPS) protects mice from Fas-mediated apoptosis, we aimed to explore if the observed protective effect is mediated by Janus kinase (JAK)/Stat3 signaling pathway.

Materials and Methods: Male C57BL/6 mice were treated with ruxolitinib, selective JAK1/2 inhibitor, (double oral gavage with 2 hours between applications, to a final dose of 180 mg/kg) or vehicle (Peg300/DMSO/dH2O) followed by intraperitoneal injection of LPS (0.025 μg/g) 2 hours after. Liver samples were harvested after 90 minutes and phosphorylated Stat3 (pStat3) and Stat3 expressions were determined (western blot). The gene expression of proinflammatory cytokines and apoptosis-related factors were analyzed (qPCR). Liver specimens were also collected from LPS or saline treated mice (2 hours after treatment) for immunohistochemistry (pStat3). To explore the possible involvement of JAK/Stat3 pathway in the anti-apoptotic effect of the inflammation, mice were treated with ruxolitinib or vehicle, followed by LPS and anti-Fas (JO2) activating antibody (0.25 μg/g, intravenously) 2 hours after LPS. Sera were collected 6 hours after anti-Fas application for ALT measurement.

Results: LPS treatment induced the phosphorylation of Stat3 in hepatocytes. Immunoblots revealed an increase in pStat3 expression in liver samples after LPS treatment, while immunohistochemical analysis showed an increase in pStat3 positive hepatocytes when compared to saline treated mice. Ruxolitinib pretreatment successfully inhibited the LPS-induced expression of pStat3 and densitometry showed significantly lower pStat3/Stat3 ratio in ruxolitinib/LPS treated mice in comparison with vehicle/LPS group (p=0.008). Finally, *in vivo* experiments showed that ruxolitinib pretreatment abrogates the anti-apoptotic effect of LPS. Ruxolitinib/LPS/anti-Fas treated mice had significantly higher levels of ALT when compared with vehicle/LPS/anti-Fas treated group (median [IQR]: 800.0 [532.5-1190] vs. 175.0 [57.5-442.5] IU/L respectively; p=0.026). Ruxolitinib did not aggravate the anti-Fas induced apoptosis as determined by ALT levels (ruxolitinib/anti-Fas vs. vehicle/anti-Fas, p=0.57). Interestingly, ruxolitinib pretreatment augmented the LPS-induced gene expression of proinflammatory cytokines (TNF-alpha, IL-1 and IL-6), and significantly reduced the expression of Bcl-xL in comparison with LPS treated mice (p=0.02).

Conclusion: Acute inflammation induced by LPS protects mice from Fas-mediated liver injury. Stat3 could be one of the crucial mediators involved in this protective effect, as ruxolitinib-induced inhibition of JAK signaling significantly reversed the anti-apoptotic feature of LPS.

THE SUBTLE CHANGE IN THE ANTIVENOM PURIFICATION PROTOCOL AFFECTS DIFFERENTLY DOWNSTREAM INTERMEDIATES AND THE FINAL PRODUCT

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Animal-derived antibody therapeutics are widely used in medicine for viral and toxin neutralization. Although the various effective manufacturing methodologies have already been implemented into commercial scale production, due to more demanding regulatory requirements there is the constant need for their improvement. One of the major challenges in optimization concerns achieving the final product of high purity in order to ensure its safety and clinical efficiency.

Recently, we have established the high yielding protocol for the preparation of snake venom-specific $F(ab')_2$ fragments from hyperimmune horse plasma. The process itself consists of two main phases: caprylic acid selective precipitation of majority of contaminating proteins and pepsin fragmentation of IgG molecules preserved in solution. In the previous research, we have noticed that the most variable part of our refining scheme was caprylic acid fractionation step, thus affecting the purity of the IgG intermediate preparation. Despite the fact that we have succeeded to obtain the $F(ab')_2$- based final product of 100% purity, we aimed for further process improvement regarding its stability by optimizing this critical step.

Our goal was to investigate the possible beneficial impact of higher caprylic acid concentration on the IgG fraction composition in order to establish less variable and more reproducible manufacturing process.

Here we showed the results indicating that even small variations in operating parameters could cumulatively affect refinement strategy for immunotherapeutics preparation, influencing consequently the quality and, particularly, safety of the final product.

INFLAMMATION AND INCREASED OSTEOCLASTOGENESIS IN OSTEOGENESIS IMPERFECTA MURINE

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Introduction: Osteogenesis imperfecta (OI) is a disease caused by defects in type I collagen production that results in brittle bones. While the pathology is mainly caused by defects in the osteoblast lineage, there is also elevated bone resorption by osteoclasts resulting in high bone turnover in severe forms of the disease. Osteoclasts originate from hematopoietic myeloid cells, however changes in hematopoiesis have not been previously documented in OI.

Aim: In this study, we evaluated hematopoietic lineage distribution and osteoclast progenitor cell (OCP) frequency in bone marrow, spleen and peripheral blood of osteogenesis imperfecta murine (OIM) mice, a model of severe OI.

Materials and Methods: OIM mice were obtained from Jackson Labs (Bar Harbor, ME) in a mixed background (B6C3Fe a / a - Col1a2 oim /J). Isolated cell suspensions were stained for multiple hematopoietic markers, including OCPs (CD3, B220, CD11b, Ter119, Ly6c, CD117, CD115), using commercially available antibodies. Osteoclasts were differentiated with RANKL and M-CSF, and defined as TRAP+ cells with 3 or more nuclei. Micro-CT was performed on femurs by the UConn Health Micro CT imaging facility with a μCT40 instrument (Scanco). Cytokines were measured with Mouse TNFα Quantikine High Sensitivity ELISA kit and Mouse IL1α Quantikine ELISA kit (R&D Systems). Murine TNFR2:Fc was administered to mice to neutralize TNFα (Amgen, Thousand Oaks, CA). OIM mice were randomly assigned to receive either TNFR2:Fc or vehicle (saline) treatment.

Results: We found splenomegaly in all ages examined, and expansion of myeloid lineage cells (CD11b+) in bone marrow and spleen of 7–9 week old male OIM animals. OIM spleens also showed an increased frequency of purified OCPS (CD3-B220-CD11b+Ly6c+CD115+). This phenotype is suggestive of chronic inflammation. Isolated OCPs from both spleen and bone marrow formed osteoclasts more rapidly than wild-type controls. We found that serum TNFα levels were increased in OIM, as was IL1α in OIM females. We targeted inflammation therapeutically by treating growing animals with murine TNFR2:Fc, a compound that blocks TNFα activity. Anti-TNFα treatment marginally decreased spleen mass in OIM females, but failed to reduce bone resorption, or improve bone parameters or fracture rate in OIM animals.

Conclusion: We have demonstrated that OIM mice have changes in their hematopoietic system, and form osteoclasts more rapidly even in the absence of OI osteoblast signals. OIM mice have indications of chronic inflammation but therapy targeting TNFα did not improve disease parameters.

ASSOCIATION BETWEEN CXCL9/10 POLYMORPHISMS AND ACUTE REJECTION OF LIVER ALLOGRAFT

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Introduction: While increased serum concentrations of CXCL9/10 are associated with the acute cellular rejection (ACR) occurrence, the association of CXCL9/10 single nucleotide polymorphisms (SNP) with ACR after liver transplantation (LT) remains unknown.

Patients and Methods: Polymorphisms of CXCL9 (rs10336) and CXCL10 (rs3921) were determined in 215 transplanted patients by PCR using the comercialyy available Taqman assays . ACR was defined as biopsy proven within 6 months after LT. Plasma concentrations of CXCL9/10 were determined in newly transplanted patients by ELISA.

Results: There were 59 patients with ACR and 156 patients without ACR. Lack of association between CXCL9/10 genotypes and incidence of ACR was found. Patients with CXCL9 genotype AA developed ACR earlier than patients with the GG genotype (p=0.003) with similar results for CXCL10 gene (CC vs GG; p=0.005). There was no statistically significant difference in plasma concentrations of CXCL9/10 between the rejectors and the non-rejectors. Patients with AA CXCL9 genotype had significantly higher CXCL9 plasma concentration than patients with AG (p=0.01) or GG genotype (p=0.045).

Conclusion: SNP of CXCL9 (rs10336) and CXCL10 (rs3921) are not associated with the incidence of ACR. However, patients with CXCL9 genotype AA developed ACR earlier and the same genotype was associated with greater plasma concentrations suggesting the involvement of CXCL9 mediated processes in ACR development.

UNDERSTANDING B-CELL CHRONIC LYMPHOCYTIC LEUKAEMIA DEVELOPMENT

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Amongst hematopoietic cancers in the Western hemisphere, B-cell chronic lymphocytic leukemia (B-CLL) is the forerunner when regarding the sheer number of newly diagnosed patients. It primarily affects people over 50 years of age and the number of patients is only expected to grow as the population ages. It is distinguished by the accumulation of a malignant clone blocked in the progression of differentiation, but, even though the disease is clonal, it is remarkably heterogenic, which accounts for the differences in patients' response to therapy and overall survival.

In order to determine the factors influencing the variability of the disease, we analyzed the occurrence of several important factors in the differentiation and maturation of B cells. We examined NOTCH1, its downstream targets DELTEX and HES1, as well as AIOLOS in a cohort of B-CLL patients on the level of mRNA and protein expression.

NOTCH1 expression was found in all but one sample, its expression being lower than in controls taken from healthy individuals. Nevertheless, it was found to be activated in all of the samples. Surprisingly, HES1, its downstream effector, was not expressed. DELTEX1 was expressed variably but to a higher degree than in controls. The most striking difference in expression was found with AIOLOS whose levels ranged from multiple times lower to multiple times higher than the control. It also could not be correlated with the protein expression, suggesting posttranslational modifications. Based on the results obtained, we conclude that the Notch pathway is noncanonically, but universally, activated in all patient samples. Both NOTCH1 and AIOLOS are variably expressed, but mutually independent. The innate heterogeneity is perhaps best emphasized with the extreme disparity of AIOLOS expression.

The search for the discriminatory trait in B-CLL continues, and may never be finished, but we made some promising advancements in understanding the mechanisms underlying the disease.

CYTOMEGALOVIRUS-ENCODED PVR REGULATOR M154 AFFECTS THE LARGEST KNOWN NUMBER OF IMMUNOLOGICALLY RELEVANT TARGETS AND IMPAIRS MCMV-SPECIFIC CD8 T CELLS

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Introduction: Cytomegaloviruses (CMVs) are known for their regulation of cellular ligands for different immune receptors in order to circumvent host immune surveillance. Both human and murine CMVs (MCMV) downregulate the surface expression of CD155 (Poliovirus receptor; PVR) molecule, which serves as a stress-induced ligand for activating receptor CD226 (DNAM-1), but also for inhibitory receptors TIGIT and CD96.

Aim: We previously characterized the MCMV-encoded glycoprotein, m20.1, as responsible for surface PVR downregulation, intracellular retention in an immature form and its commitment to proteolytic degradation. We also showed that this m20.1 function is to interfere with PVR-DNAM-1 activating immune pathway, essential in the early control of CMV. By screening a panel of MCMV deletion mutants, we identified another MCMV product, m154, as a second regulator of PVR. It was shown previously that m154 targets another ligand, CD48, suggesting its broader effect on immune response. Goal of this research was to decipher the mechanism of action of m154 protein.

Materials and Methods: Characterization of m154 function involved analysis of the mechanism of PVR downregulation on murine embryonic fibroblasts or antigen presenting cells (qPCR, flow cytometry, immunofluorescence; IF)), analysis of the kinetics of expression (Western blot, IF) and localization of m154 in infected cells (IF), identification of other potentially relevant targets of m154 (construction of Δm154 viral mutant, flow cytometry), and analysis of the function of Δm154 virus on immune response *in vitro* (T-cell stimulation assay) and *in vivo* (organ titration from infected mice, depletion of immune cell populations).

Results: Our results revealed that m154 acts postranscriptionally and is involved in the active removal of PVR from the cell surface. Upon infection, m154 localizes at the plasma membrane and in trans-Golgi network, interfering with mature PVR endocytosis via AP-1 compartment and redirecting PVR to the lysosomal degradation. In addition, we showed that m154 perturbs surface expression of several other immunologically relevant molecules, many of which act as T-cell costimulatory signals. As a result of these immunoevasive properties of m154, antigen presenting cells infected with viral mutant lacking m154 show superior *in vitro* priming capacity and the Δm154 mutant is attenuated *in vivo*, which depends both on NK and CD8 T cells.

Conclusion: Our findings strongly suggest that m154, by interfering with surface expression of multiple immunologically relevant molecules, has a prominent role in viral escape from the host immune surveillance. However, additional research is needed to examine what is common to the molecules that m154 is targeting.

THE EXPRESSION LEVEL CHANGES OF LINEAGE-SPECIFIC CELL SURFACE MOLECULES ON INNATE IMMUNE CELLS IN RESPONSE TO ORTHOHANTAVIRUS INFECTION

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Introduction: Puumala and Tula orthohantavirus are enveloped RNA viruses, belonging to genus *Orthohantavirus* (HTV), family *Hantaviridae*, order *Bunyavirales*. Puumala orthohantavirus (PUUV) is pathogenic causing mild to moderate forms of hemorrhagic fever with renal syndrome in Euroasia while Tula orthohantavirus (TULV) is considered apathogenic due to limited evidence of pathogenesis in humans. Monocytes, macrophages and dendritic cells (DC), as innate immune system cells, are target cells for HTV potentially contributing to dissemination of the virus in the body and development of the disease. There is still a lack of knowledge about the mechanisms of immune response to HTV. The aim of this *in vitro* study was to analyze the expression dymanics of the selected cell surface molecules on monocytes, macrophages and DC infected with PUUV or TULV in order to investigate whether orthohantavirus infection triggers differentiation and subsequent cell polarization.

Materials and Methods: Primary human monocytes were infected *in vitro* with PUUV or TULV and cultured for seven days post infection. Immunophenotyping was done in three time points using in-house created polychromatic screening panel with antibodies specific for surface molecules on monocytes, macrophages and DC. After staining and fixation, samples acquisition was performed on a Navios flow cytometer (Beckman Coulter, USA). Data files were analysed using FlowLogic software (Inivai Technologies, Australia).

Results: The expression levels of lineage-specific (monocyte, macrophage and DC) cell surface molecules between orthohantavirus infected cells and mock infection were different, as well as between pathogenic and apathogenic infections.

Conclusion: Orthohantaviruses induce differentiation of primary human monocytes as seen at the level of cell surface markers expression. Differences are observed between pathogenic and apathogenic orthohantaviruses infections.

ABBERANCIES OF SPECIFIC PERIPHERAL BLOOD T-CELL AND MONOCYTE SUBPOPULATIONS IN ANKYLOSING SPONDILITIS CORRELATE WITH DISEASE ACTIVITY

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Introduction: Ankylosing spondylitis (AS) is associated with abnormal immune cell functions, including T-cells and monocytes. Although several recent studies stressed role of T-cells and monocyte subpopulations in AS, these mechanisms are significantly less understood compared to other rheumatic autoimmune diseases.

Aim: We aimed to compare the frequency of T-cell and monocyte subpopulations between AS patients and controls, and to correlate them with disease activity.

Materials and Methods: Mononuclear cells were isolated from peripheral blood of healthy controls (CTRL) (n=110) and AS (n=65) patients. T-cell and monocyte phenotype was determined using flow cytometry for following markers: Th1/2 (CD3+CD4+CCR6-), Th17 (CD3+CD4+CCR4+CCR6+), Tfh (CD3+CD4+CXCR5+), Tc (CD3+CD8+), memory Tc (CD3+CD8+CCR4+) double-positive (dp) Tc (CD3+CD4+CD8+); and monocytes (CD3-CD19-CD56-CD11b+CD14+). On lymphocytes, activation markers (IL21R, CD25) and migration (CD11b) were analyzed; on monocytes, chemokine receptors CCR1, CCR2, CCR4 and CXCR4, as well as RANK were assessed. Frequencies of analyzed subpopulations were correlated with clinical parameters, including ASDAS (Ankylosing Spondylitis Disease Activity Score), BASDAI (Bath Ankylosing Spondylitis Disease Activity Index), VAS assessments of pain in spine and peripheral joints, stiffness intensity and duration, erythrocytes sedimentation rate and C-reactive protein.

Results: Tfh were decreased (16.9 [13.18-20.20] CTRL vs 12.35 [9.61-16.00] AS, p=0.038), correlating negatively with physician's assessment of disease activity (rho=-0.767, p=0.016). Memory Tc were also decreased (10.8 [8.04-14.13] CTRL vs 8.14 (3.01-10.34) AS, p=0.022). Frequency of dp Tc negatively correlated with BASDAI (rh= -0.65, p=0.029) and pain assessment (rho=-0.791, p=0.004). Frequency of monocytes was decreased (13.75 [11.44-19.36] CTRL vs 9.92 [6.33-16.79] AS, p<0.001), while subpopulation expressing RANK was increased (6.15 [3.68-9.96] CTRL vs 11.86 [6.37-22.17] AS, p=0.007). In monocytic subpopulations expressing chemokine receptors, CCR1 correlated with BASDAI (rho=0.524, p 0.037) and pain assessment (rho=0.565, p=0.023); CCR2 correlated with stiffness duration (rho=0.676, p 0.011); CXCR4 expression was increased (3.89 [2.42-7.5] CTRL vs 10.49 [7.53-16.63] AS, p<0.001) and correlated with BASDAI (rho=0.521, p=0.039) and stiffness intensity (rho 0.600 p=0.014).

Conclusion: Results show changes in T-cells and monocyte subpopulations induced in AS and indicate their possible importance for AS pathogenesis. Peripheral blood dp Tc cells may be of particular interest as further research targets for novel therapeutic approaches, since their frequency is associated with disease activity, as well as patient's pain assessment. Function of dp Tc in human diseases is still controversial as reported potent immune-suppressors or as cells with high cytotoxic potential.

ALLERGY TO *OLEACEAE* IN MEDITERRANEAN COUNTRIES-IS IT A PROBLEM IN CROATIA?

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Introduction: Trees from the family *Oleaceae* are among the most important causes of respiratory allergy in Mediterranean countries. *Oleaceae* familiy comprises 4 genera: olive (*Olea europea*), ash (*Fraxinus excelisior*), lilac (*Syringa vulgaris*) and common privet (*Ligustrum vulgare*). Olive pollenosis ranges from 30-40% in Italy up to 80% in Spain, while ash is considerred very important in central European countries. *Oleaceae* trees are highly cross-reactive. Studies have shown major olive allergen in Spanish population Ole e 1 to be highly homologous to the major ash allergen Fra e 1. People sensitized to *Oleaceae* pollen could present allergenic reactions during winter (due to ash pollen allergens), spring (caused by the olive pollen allergens) and summer (as consequence of the Ligustrum or lilac flowering). While olive pollen is the first cause of pollinosis in several Spanish and Italian countries, the exact prevalence of *Oleaceae* pollen sensitisation in Croatia has not been investigated so far.

Aim: The aim of the study was to explore the prevalence of skin sensibilization to olive and possible cross reactivity with ash pollen in schoolchildren from continental part of Croatia (city of Zagreb).

Materials and Methods: 85 randomly selected schoolchildren aged 7-14 yeras old from the city of Zagreb were included in the study. Skin prick tests with the olive and ash extracts (Bencard®) were performed using standard procedure. The result was considerred positive if the diameter of the wheal was 3 mm or greater. A histamine solution 10 mg/ml was used a positive control. Negative control was used as well. Antihistamines and skin rash at the testing area were the exclusion criteria.

Results: We included a total number of 85 children aged 7-14 years old. Total prevalence of positive skin tests to olive was 7% and all of them were positive to ash as well (7%), with no difference among boys and girls. All of them were also sensitized to other pollen allergens, mostly grasses and ragweed.

Conclusion: According to our study, sensibilization rate to olive and ash extract in our sample was 7%. We suppose that olive and ash pollen show high cross-reactivity pattern in our population as in Spain and Italy, although the prevalence of sensibilization might be lower than in other Mediterranean countries. For further conclusions, we should do the tests in the costal part of Croatia and do the molecular analysis. All patients allergic to ash should be aware of possible cross-reactive pollenosis while travelling along the coast.

Author Index

PUBLISHER
Croatian Immunological Society

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PRINT
Cerovski d.o.o.

FRONTPAGE PHOTOGRAPH
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