2017 ANNUAL MEETING OF THE
CROATIAN IMMUNOLOGICAL SOCIETY WITH EFIS ON TOUR
ZAGREB, OCTOBER 20th-21st 2017

ORGANIZED BY
CROATIAN IMMUNOLOGICAL SOCIETY
EUROPEAN FEDERATION OF IMMUNOLOGICAL SOCIETIES
University of Zagreb School of Medicine
University of Rijeka Faculty of Medicine

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Croatian Immunological Society                                                                 |
| 14:15-14:45  | EFIS ON TOUR             | René A.W. van Lier
Sanquin Blood Supply Foundation, Amsterdam, Netherlands
Blood and beyond: properties of human tissue-resident T cells |
| 14:45-15:15  | Vanda Juranić Lisnić     | Faculty of Medicine, University of Rijeka, Croatia
Complex interplay between Ly49 receptors and murine cytomegalovirus |
| 15:15-15:45  | Lorenzo Moretta          | Ospedale Pediatrico Bambino Gesù, Rome, Italy
Human NK cells: from biology to clinical applications |
| 15:45-16:15  | Danka Grčević            | University of Zagreb School of Medicine, Croatia
Osteoclasts: on the crossroad between immune and bone systems |
<p>| 16:15-16:45  | COFFEE BREAK             |                                                                                     |</p>
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<td>Winfried Pickl</td>
<td>Center for Pathophysiology, Infectiology &amp; Immunology,</td>
<td>Genetic restriction of antigen-presentation dictates allergic sensitization and disease in humanized mice: Novel ways to modulate allergy by targeting T regulatory cells</td>
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<td>Felix Wensveen</td>
<td>Faculty of Medicine, University of Rijeka, Croatia</td>
<td>The weak will not perish; molecular mechanisms controlling clonal selection of effector and memory lymphocyte populations</td>
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<td>17:45-18:15</td>
<td>Günnur Deniz</td>
<td>Istanbul University, Turkey</td>
<td>Regulatory Natural Killer Cells</td>
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<td>Andreas Radbruch</td>
<td>Deutsches Rheuma-Forschungszentrum, Berlin, Germany</td>
<td>Maintenance of immunological memory - cycling and circulating versus resting and resident memory cells</td>
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EFIS ON TOUR (continued)

*Chairs*: Danka Grčević and Pablo Engel
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<td>08:30-09:15</td>
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<td>Jenny Henzen</td>
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<td>MEETING OF CROATIAN IMMUNOLOGICAL SOCIETY - SESSION I</td>
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<td>Ilija Brizić</td>
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<td>Faculty of Medicine, University of Rijeka, Croatia</td>
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<td>Brain-resident memory CD8 T cells induced by congenital CMV infection</td>
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<td>prevent brain pathology and virus reactivation</td>
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<td>Inga Kavazović: Eomes broadens the scope of CD8 T cell memory by inhibiting apoptosis in low-affinity cells</td>
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<td>Kristina Vuković: KLRG1+ CD8 T cells induced by cytomegalovirus vector expressing RAE-1γ ensure the outstanding protection against tumor challenge</td>
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<td>Vedrana Jelenčić: NKG2D sets thresholds for specific activating receptors early in NK cell-development</td>
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<td>Daria Kveštak: NK cells-derived interferon γ mediate microglia polarization and delay in cerebellar growth during congenital CMV infection</td>
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<td>10:30-12:00</td>
<td><strong>POSTER SESSION with REFRESHMENTS</strong>&lt;br&gt; <em>Chairs: Alemka Markotić, Tomislav Kelava, Felix Wensveen</em></td>
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<td><strong>MEETING OF CROATIAN IMMUNOLOGICAL SOCIETY - SESSION II</strong>&lt;br&gt; <em>Chairs: Asja Stipić Marković and Vanda Juranić Lisnić</em></td>
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<td>12:30-14:15</td>
<td><strong>SELECTED ORAL PRESENTATIONS</strong>&lt;br&gt;<strong>Marko Šestan:</strong> Virus-induced IFNγ causes insulin resistance in muscle and derails glycemic control in obesity&lt;br&gt;<strong>Sonja Marinović:</strong> The role of innate immune cells in development of NAFLD&lt;br&gt;<strong>Tamara Gulić:</strong> Molecule 1, selectively expressed in M2-like Macrophages affecting monocytes motility&lt;br&gt;<strong>Ivana Stražić Geljić:</strong> Cytomegalovirus-encoded protein m154 affects an immunologically relevant ligand CD155</td>
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| 14:15-14:45 | **MEETING OF CROATIAN IMMUNOLOGICAL SOCIETY - SESSION III**  
|        | **Chairs:** Alenka Gagro and Bojan Polić  
|        | **INVITED LECTURE**  
|        | Barbaros Oral  
|        | Uludag University, Bursa, Turkey  
|        | Indoleamine-2,3-dioxygenase (IDO) for the treatment of arthritis                       |
| 14:45-15:30 | **SELECTED ORAL PRESENTATIONS**  
|        | Alan Šućur: Chemokine signals are crucial for enhanced homing and differentiation of circulating osteoclast progenitor cells  
|        | Lovro Lamot: Hypermethylation of NLRP3 promoter region could be responsible for decreased gene expression, inflammasome malfunction and gut dysbiosis in early phase juvenile spondyloarthritis  
|        | Maja Ledinski: Analysis of intestine microbiome in the Alzheimer's disease rat model  
|        | Marija Maskalan: The impact of HLA-DPB1 matching in hematopoietic stem cell transplantation |
| 15:30-16:00 | **INVITED LECTURES**  
|        | Ihsan Gursel  
|        | Bilkent University, Ankara, Turkey  
|        | Exosome-based Cancer Vaccines  
| 16:00-16:30 | Alemka Markotić  
|        | Clinic for Infectious Diseases “Fran Mihaljević”, Zagreb, Croatia  
|        | Immunoreactions to hantaviruses                                                           |
| 16:30-16:45 | **CLOSING REMARKS AND POSTER AWARDS**  
|        | Danka Grčević, president  
|        | Croatian Immunological Society                                                            |
| 16:45-17:00 | **DEPARTING COFFEE & SNACKS**                                                            |
| 17:00-TBD  | **SIGHTSEEING TOUR**                                                                     |
LECTURES
Protection against respiratory infections in the lung is mediated by tissue-resident memory T-cells (TRM). We characterized memory T-cells from human lungs through transcriptome and functional analyses and revealed the existence of two distinct, but related memory T-cell populations in lung tissue. We discovered two transcriptional pathways that play essential roles the maintenance of TRM in tissue: one under the influence of NOTCH signaling and a second one controlled by the homologous transcriptional repressor BLIMP-1 and HOBIT. Further, consistent with a requirement for prompt responsiveness to prevent microbial colonization of the respiratory barrier tissue, lung TRM constitutively transcribe deployment ready mRNAs encoding effector molecules and produce effector proteins with accelerated kinetics in response to TCR activation.


Cytomegaloviruses downregulate MHC I from the cell surface in order to avoid recognition by T cells. Since this strategy could trigger the NK cell-mediated “missing-self” recognition, murine cytomegalovirus (MCMV) encodes m04, a protein which brings a portion of MHC I molecules back to the cell surface enabling them to engage inhibitory Ly49 receptors (iLy49s). 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. In certain cases, Ly49 receptors recognize MHC I, m04 and an additional, 11kDa viral protein encoded by the MCMV’s most abundant transcript (MAT), which also encodes another protein of unknown function and contains a binding site for cellular micro RNA (Marcinowski et al (2012) PLOS Pathogens; Juranic Lisnic et al (2013) PLOS Pathogens). Here we show that the deletion of MAT results in a modest downregulation of MHC I from the cell surface but drastic changes the repertoire of host and viral peptides presented in MHC I. In addition, viral mutants lacking MAT are attenuated in vivo in NK cell-dependent manner.
NK cells play an important role in innate defenses against viruses and tumors. They are part of a recently identified family of innate lymphoid cells (ILC). All ILC do not express receptors encoded by rearranging genes. Remarkably, both the transcription factors involved in ILC development from hemopoietic precursors and their functional properties mirror those of well-defined T cell subsets. While NK cells display a potent anti-tumor activity in vitro and participate in the immunosurveillance against tumors and in the control of metastatic spreading, the tumor microenvironment may sharply hamper their functional capability. This effect is primarily related to downregulation of the surface expression of activating receptors involved in tumor cell recognition and killing. Another inhibitory mechanism mediated by PD-1 – PD-L1 interaction may severely compromise the NK cell-mediated anti-tumor activity. Importantly, the NK cytolytic activity can be restored by anti-PD1 mAbs. These data are particularly relevant for those tumors that have lost/downregulated the HLA-class I expression, thus escaping the CTL-mediated control. NK cells have been exploited in the haploidentical hemopoietic stem cells transplantation (HSCT) to cure acute high-risk leukemias (originally applied when no HLA-compatible donors were available). The infusion of mega-doses of T-depleted CD34+ HSC allows an efficient engraftment with unfrequent, mild grade, GvHD. In haplo-HSCT, in the absence of donor T lymphocytes, NK cells play a central role in the anti-leukemia effect. More recently, we applied a new strategy of haplo-HSCT, based on the infusion of TCRαβ- and CD19-depleted mononuclear cells that include, in addition to CD34+ cells, mature donor NK cells and TCRγδ+ T cells. This graft manipulation led to a further major improvement of the clinical outcome of pediatric patients, particularly in AML, with a 5 years overall survival of ~70% in both ALL and AML.
OSTEOCLASTS: ON THE CROSSROAD BETWEEN IMMUNE AND BONE SYSTEMS

Danka Grčević

University of Zagreb School of Medicine, Zagreb, Croatia

Osteoclasts are multinuclear cells with the unique ability to resorb bone. They originate from hematopoietic progenitors of monocyte/macrophage lineage, expressing receptors for the two crucial osteoclastogenic factors: macrophage colony-stimulating factor (M-CSF) receptor (CD115, cFms) and receptor activator of nuclear factor-κB (RANK). Furthermore, elevated levels of a wide range of proinflammatory cytokines (interleukin (IL)-1, IL-6, IL-15, IL-17, IL-18, IL-21, IL-22, IL-23, tumor necrosis factor α (TNF-α)) and chemokines (CCL2, CCL3, CCL4, CCL5) act in synergy to M-CSF/RANK signals to promote osteoclastogenesis. The inflammatory milieu favors recruitment and activation of osteoclasts, and leads to bone destruction as a serious complication associated with arthritis and with other inflammatory processes. Osteoclast progenitors are physiologically found in the bone marrow as well as in the circulation, so they could be recruited to the sites of inflammation by various chemotactic signals. The frequency and activity of osteoclast progenitors correspond to arthritis severity, and may be used to monitor disease progression and bone resorption. However, molecular signals underlying systemic activation and attraction of osteoclast progenitors to articular and extra-articular bone surfaces under the inflammatory conditions are not precisely defined. Our group has long been studied functional activity of different osteoclast progenitor subpopulations and signals important for their migration to bone lesions, and subsequent local and systemic bone resorption during the course of collagen-induced arthritis in mice as well as in different forms of chronic joint diseases in humans. In the mouse model of arthritis, we were able to identify potent osteoclastogenic progenitor subpopulation specifically expanded in arthritis, bearing the phenotype CD45+CD3-B220-NK1.1-Ly6G-CD11b+CD115+, highly susceptible to CCL2 and CX3CR1 signals. This subpopulation has been shown to increasingly recirculate and home to the sites of inflammation in vivo, and to produce fully mature osteoclasts in vitro. In human studies, a subpopulation of peripheral blood monocytes (CD45+CD3+CD19+CD56+CD11b+CD14+) from arthritic patients effectively forms bone-resorbing osteoclasts and responds to CCL5 and CXCL10 chemokines by enhanced migration and maturation. Control of the activity and migratory behavior of osteoclast progenitors as well as interruption of crucial bone/joint chemotactic signals represent promising therapeutic targets in arthritis.

Funded by the Croatian Science Foundation, project number 5699.
IgE-associated allergies result from misguided immune responses against innocuous antigens. CD4+ T lymphocytes are critical for initiating and perpetuating that process, yet the crucial factors determining whether an individual becomes sensitized towards a particular allergen remain unknown. We here created a novel human TCR and HLA-DR1 (TCR/DR1) transgenic mouse model of asthma, based on the human-relevant major mugwort pollen allergen to examine these critical factors upon natural allergen exposure via the airways in the absence of systemic priming and adjuvants. Acute allergen exposure led to IgE-independent airway hyperreactivity (AHR) and Th2-prone lung inflammation in TCR/DR1, but not DR1, TCR or WT control mice, that was alleviated by prophylactic IL-2/αIL-2 mAb complex-induced expansion of Tregs. Chronic allergen exposure sensitized one third of single DR1 transgenic mice, however, without impacting on lung function. Similar treatment led to AHR and Th2-driven lung pathology in >90% of TCR/DR1 mice. Prophylactic and therapeutic expansion of Tregs with IL-2/αIL-2 mAb complexes blocked the generation and boosting of allergen-specific IgE associated with chronic allergen exposure. Moreover, we show that treatment with virus-like nanoparticles harboring full length major mugwort pollen allergen and representing a non-allergenic, non-sensitizing platform, prevents allergic sensitization by increasing lung-resident Treg numbers. We identify genetic restriction of allergen presentation as key factor in allergic sensitization and confirm the importance of the balance between allergen-specific T effector and Treg cells for modulating allergic immune responses.

*Funded by the Austrian Science Fund (FWF) SFB F4609, DK-W1248.*
THE WEAK WILL NOT PERISH; MOLECULAR MECHANISMS CONTROLLING CLONAL SELECTION OF EFFECTOR AND MEMORY LYMPHOCYTE POPULATIONS

Felix Wensveen
Faculty of Medicine, University of Rijeka, Croatia

The adaptive immune system consists of millions of clones, each unique based on its antigen receptor. Upon antigen-encounter, antigen-specificity is the primary determining factor whether an activated cell is allowed to survive and contribute to the effector and memory cell pools. However, selective criteria must differ between effector and memory populations. Effector cells are faced with an acute infection and therefore benefit from stringent selection of cells with high specificity. Memory cells must anticipate re-infection with a pathogen that has undergone mutations in its immuno-dominant epitopes in order to prevent recognition by the immune system. Affinity-based memory selection must therefore allow survival of more clones, in order to sample a larger fraction of the potential pathogen-carried sequence space. The molecular mechanisms underlying affinity-based selection are only now starting to be understood.

Formation of both the effector CD8 T and B cell pools involves a Darwinian struggle for pro-survival cytokines. CD8 T cell clones of high-affinity induce higher levels of the IL-2 receptor. As a result, they sustain higher-levels of the pro-survival protein Mcl-1, providing them with a survival advantage over low-affinity clones. Activated B cells use a highly similar mechanism, as they induce the receptor for BAFF, a cytokine which also mediates Mcl-1 stabilization, in an antigen-dependent fashion. Genetic ablation of Noxa, the natural antagonist of Mcl-1, results in reduced selective pressure and survival of low-affinity clones into the effector CD8 and B cell pools. Noxa deficient mice therefore have effector CD8 and B cell pools of overall lower affinity and are able to cope with acute infections with reduced efficiency.

Memory cells are faced with the same selective struggle for external pro-survival signals. However, we find that CD8 memory T cell precursors have a survival advantage within a window of signal intensity, defined by the affinity and the avidity of the activating stimulus. Within this window, induction of the transcription factor Eomes drives expression of the pro-survival protein Bcl-2. At higher signal intensities, Eomes-mediated Bcl-2 induction is suppressed by T-Bet. Genetic ablation of Eomes results in loss of low-affinity memory cells and a reduced ability to fight mutated pathogens. Thus, during memory formation, differences in intrinsic survival capacity result in reduced clonal selection in a struggle for external survival factors.

This lecture will provide a comprehensive overview of clonal selection strategies for effector and memory lymphocyte populations.
REGULATORY NATURAL KILLER CELLS

Günnur Deniz

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Natural killer (NK) cells are a subset of lymphocytes that principally participate in innate immunity but may also have important roles in determining the outcome of the adaptive immune responses. In human they are phenotypically defined as CD3-CD56+ lymphocytes, and the major functional properties of NK cells are cytotoxicity, cytokine production and contact dependent co-stimulation.

NK cell regulatory capabilities mediated by cytokine and chemokine release, they exert their activity by producing high amount of IFN-γ, that activates a strong inflammatory response. Indeed, other than IFN-γ, NK cells are able to produce many other important cytokines and chemokines, including myeloid differentiation and activation factors such as IL-3, GM-CSF, TNF-α, IL-5, IL-13, IL-10 and chemokines such as MIP-1, RANTES and IL-8.

Similar to the Th1 and Th2 subsets of CD4+ T cells, NK cells are also divided into NK1 and NK2 subpopulations according to the profile of cytokine secretion. Our and other results showed that in the presence of IL-12 or IL-4, human NK cells could differentiate into NK cell subsets secreting distinct cytokine patterns similar to T cells. NK cells grown in IL-12 (NK1) produce predominantly IFN-γ, whereas NK cells grown in IL-4 (NK2) produce IL-5 and IL-13. Although these NK cell subsets do not differ in cytotoxic activity, NK1 cells express higher levels of cell surface CD95 (Fas) antigen than NK2 cells and are more sensitive to antibody or chemically induced apoptosis. It has been showed that the type 2 cytokines produced by NK2 cells are dominant in asthma and tumor microenvironment and are involved in pathogenesis of asthma and cancer. The IL-10- and TGF-α-secreting NK3 type cells also play major roles in immune regulation and promote transplant and pregnancy tolerance. Similar to suppression of both cytokine production and antigen-specific proliferation of Th1 and Th2 cells by IL-10, our results showed that IL-10 secreting NK cells suppress both allergen-stimulated T cells and PPD-stimulated T cells, whereas IFN-γ secreting NK cells did not show any suppression. The findings suggest that in vivo existence of a regulatory NK cell subset, which indeed may play an immune regulatory and suppressor role.
MAINTENANCE OF IMMUNOLOGICAL MEMORY - CYCLING AND CIRCULATING VERSUS RESTING AND RESIDENT MEMORY CELLS

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Conventional concepts of memory postulate that antigen-experienced memory B and T lymphocytes circulate through the body, in quest for their antigen. Their numbers are maintained by a balance of death and homeostatic proliferation. Plasma cells are shortlived and constantly generated again from memory B cells. This conventional picture of a circulating, restless memory has been challenged fundamentally recently, by the discovery of memory lymphocytes resting in the tissues, in particular in the bone marrow. In 1997 we had discovered longlived "memory" plasma cells, which are maintained in the bone marrow. These memory plasma cells rest in terms of proliferation and migration. Reticular stromal cells organize survival niches for memory plasma cells, by providing essential survival signals, by cell contact, and by attracting accessory cells providing the essential survival factor APRIL. We have now analysed the mechanisms of their maintenance in an artificial niche ex vivo in molecular detail. We find also resident memory T and B lymphocytes in the bone marrow, and have analysed their repertoire, maintenance and reactivation. Contrary to prevailing concepts of memory maintenance by homeostatic proliferation, we could show that all CD8 memory T cells of the bone and 50% of those of the spleen are maintained as resting and resident cells, in dedicated niches organized by stromal cells.
BRAIN-RESIDENT MEMORY CD8 T CELLS INDUCED BY CONGENITAL CMV INFECTION PREVENT BRAIN PATHOLOGY AND VIRUS REACTIVATION

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Congenital HCMV infection is a leading infectious cause of long-term neurodevelopmental sequelae, including mental retardation and sensorineural hearing loss. Infection of newborn mice with MCMV intraperitoneally is a well-established model of congenital HCMV infection, which best recapitulates the hematogenous route of virus spread to brain and subsequent pathology. MCMV infection in newborn mice induces a strong inflammatory response in the brain characterized by the activation of microglia, recruitment of activated peripheral immune cells and the expression of pro-inflammatory cytokines. We set out to investigate the role, dynamics and phenotype of CD8 T cells in brain following infection of newborn mice. We show that CD8 T cells infiltrate the brain and form a pool of tissue-resident memory T cells that persist for lifetime. Adoptive transfer of naive virus-specific CD8 T cells into newborn mice reduced the virus load, prevented brain pathology, and established tissue-resident memory cells. Brain CD8 T tissue-resident memory cells were long-lived, slowly proliferating cells able to efficiently respond to challenge infection. Importantly, brain CD8 T tissue-resident memory cells controlled latent MCMV infection and their depletion resulted in virus reactivation accompanied by enhanced proinflammatory state of microglia.
AUTOIMMUNITY AND IMMUNODEFICIENCY – TWO SIDES OF THE SAME COIN?

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Immunodeficiency and autoimmunity previously were considered as mutually exclusive diseases. The increasing understanding of the complex immune regulatory and signaling mechanisms and expanding knowledge and application of genetics revealed more and more interrelationships between primary immunodeficiency syndromes and various autoimmune diseases. On one hand we find single gene defects in rare diseases presenting with autoimmunity symptoms, on the other hand there are rheumatic diseases due to polygenic traits often involving identical genes like those in primary immunodeficiency (PID). Autoimmunity and primary immunodeficiency diseases therefore may be considered as two sides of the same coin. Here we present a number of immunodeficiency diseases, their pathogenetic mechanisms and relationship to autoimmune diseases such as RAG1/RAG2 deficiencies, CTLA/LRBA deficiencies, NFκB1/2 deficiencies, their pathogenetic relationships and the clinical pictures. A better definition of the pathogenesis now allows a more specific therapeutic approach, a personalized treatment which at the same time could cure immunodeficiency as well as autoimmune disease. In summary the growing knowledge of various new mutations in immunodeficiency patients with autoimmune syndrome opens up new avenues for molecular therapy.
Rheumatoid arthritis (RA) is an inflammatory autoimmune disease that causes chronic pain and joint destruction. While drugs to treat RA exist, better treatments strategies are needed to prevent and treat this debilitating disease. As with a variety of autoimmune disorders, there is evidence of an altered level of tryptophan catabolism in RA patients and a crucial role for IDO in the induction of immune tolerance. This is indicative of activation of the enzyme indoleamine-2,3-dioxygenase (IDO), which initiates the breakdown of tryptophan. IDO is an intracellular enzyme that catalyzes the initial rate-limiting step in tryptophan degradation along the kynurenine pathway. Tryptophan starvation by IDO consumption inhibits T-cell activation, whereas products of tryptophan catabolism, such as kynurenine derivatives and oxygen free radicals, regulate T-cell proliferation and survival. IDO is widely expressed in human tissues and cell subsets and is induced during inflammation by IFN-γ and other inflammatory cytokines. Recent works have demonstrated a crucial role for IDO in the induction of immune tolerance during infection, pregnancy, transplantation, autoimmunity, and neoplasias, including hematologic malignancies.

In our study, we investigated the treatment responses of disease-inducible IDO gene overexpression in CIA mice. Initially, we demonstrated that hIDO was significantly increased following stimulation with IL-1β in HeLa cells transfected with pELAM-1pro/hIDO. Reduction of ankle thickness, clinical score and histopathological score were observed in mice receiving the disease-inducible IDO expressing vectors when compared with mock plasmid delivered mice. Also, it was shown that CD4+ T cell and CD68+ synovial macrophage ratio were diminished and IDO levels were increased following the treatment.

The results from this study demonstrate that conditional targeting IDO has the exciting potential to lead to a new approach for the treatment of RA.
The full therapeutic breadth of exosomes is hampered due to lack of exogenous ligand loading strategies within the exosome lumen. Herein, a lyophilization technique was exploited to load agents on-demand within purified exosomes. The method allows simultaneous encapsulation of multiple bio-molecular cargo ranging from oligonucleotides to protein antigen to lipidic adjuvant upon controlled reconstitution of the lyophilized mixture. The oligonucleotide loading efficiency reached 80.5±6.5% without altering fundamental exosome properties. Encapsulation into exosomes protected cargo from nuclease degradation and elicited a copious inflammatory response. Immunizing animals with exosome vaccine generated magnified and persistent Th1-biased antigen-specific IgG as well as cytotoxic T-cell responses that were essential to protect mice either from T-cell thymoma six months after vaccine administration or regressed established melanoma. While the full potential of exosome-based therapeutics remains to be defined, this work present evidence that spur the development of a highly efficient encapsulation strategy thus allows testing of targeted exosome therapeutics for human diseases in the clinic.
IMMUNOREACTIONS TO HANTAVIRUSES

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Hantaviruses (HTV), family Bunyaviridae, are emerging, enveloped RNA viruses, which cause hemorrhagic fever with renal syndrome (HFRS) in Europe and Asia and hantavirus cardiopulmonary syndrome (HPS) in Americas. In Europe, two main HTV cause HFRS: Puuma (PUUV) and Dobrava (DOBV). Reservoirs for HTV are small rodents with persistent infection and no manifest disease. A broad spectrum of clinical conditions has been recognized, ranging from inapparent or mild to a fulminant hemorrhagic process with severe renal or cardiopulmonary failure and death. There is still a significant lack of knowledge about the immune response to HTV. The absence of an animal model additionally hinders HTV research. So far, no effective antivirals, vaccines, or immunotherapeutics exist for HFRS/HPS. The strong proinflammatory response during acute infection in humans is probably responsible for viral clearance but also for potentially fatal proinflammatory-mediated disease. However, our recent data indicate possible initial immunosuppression in HFRS patients. Innate immunity is the first line of defense to various pathogens. Although monocytes/macrophages (Mo/Ma) are important immune cells in innate immunity there is still big gap in the knowledge about their role in HTV infection. They can serve as a long-term storage of HTV and their dissemination during HFRS. Infection of primary human Mo/Ma with PUUV showed low production of IFN-α. Our study showed that in primary monocytes, pathogenic HTV elicited TNF-α, GM-CSF and some β-chemokines. Differentiation of Mo/Ma increases the susceptibility to PUUV and suggests that after differentiation to tissue macrophages they might function in the spread of the virus during PUUV infection. We observed also in infected Mo/Ma differentiation toward dendritic cells phenotype in the low percentage of cells. Recent evidence suggests that specific subsets of mouse NK cells can develop long-lived and highly specific memory to a variety of antigens. In recent report the expansion of a subset of CD94/NKG2C+ NK cells in patients infected with HTV was described. This NK cell subset was functional and its expansion was maintained at high levels for a prolonged period of time after infection. Also, our ongoing study on HFRS patients infected with PUUV, shows that certain miRNAs, related to innate and adaptive immune response, may serve as a potential important biomarkers of HFRS severity. A strong T cell response involving CD8+ cytotoxic lymphocytes, and CD4+ T cells, are registered in acute HFRS. Altogether, these observations point to the complexity of the mechanisms by which HTV interfere with the innate and adaptive immunity.
ORAL PRESENTATIONS
**EOMES BROADENS THE SCOPE OF CD8 T CELL MEMORY BY INHIBITING APOPTOSIS IN LOW-AFFINITY CELLS**


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**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 immunodominant 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 both high- and low-affinity are selected into 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<sup>fl/fl</sup>CD4Cre), we showed a crucial role of Eomes for the survival of low-affinity memory cells through induction of Bcl-2. Moreover, 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 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.

**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.
KLRG1+ CD8 T CELLS INDUCED BY CYTOMEGALOVIRUS VECTOR EXPRESSING RAE-1γ ENSURE THE OUTSTANDING PROTECTION AGAINST TUMOR CHALLENGE

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Introduction. Employment of CD8 T cell therapy has expanded in recent years and reached significant success in the treatment of certain types of tumors. Moreover, combination with the blockade of immune checkpoints has proven to be very efficient in overcoming immunosuppressive tumor-microenvironment and restoring the functionality of tumor-infiltrating CD8 T cells, resulting in better tumor control and positive outcome in patients. However, effective anti-tumor CD8 T cell vaccine is still considered as a great challenge. Cytomegalovirus (CMV) is particularly attractive candidate for the CD8 T cell vaccine vector against various pathogens and tumors since it establishes lifelong persistence and ensures sustained reservoir of CMV-specific CD8 T cells. In our previous study we have shown that highly attenuated murine CMV (MCMV) coexpressing NKG2D ligand RAE-1γ (RAE-1γMCMV) and foreign CD8 T cell epitope induces highly protective and long-lasting CD8 T cell response against bacterial infection (Trsan et al. 2013).

Aim. The purpose of this study was to investigate the capacity of RAE-1γMCMV vector to serve as CD8 T cell-based anti-tumor vaccine.

Materials and Methods. We have constructed RAE-1γMCMV vector expressing ovalbumin-derived SIINFEKL epitope and investigated its potential to serve as a anti-tumor vaccine in the model of B16 melanoma expressing ovalbumin. As a control vector we used the wild-type MCMV expressing SIINFEKL epitope only. CD8 T cell response was analyzed for their effector properties and further modified by the administration of anti-TIGIT and anti-PD1 antibodies. The anti-tumor capacity was assessed in adult and newborn mice, in prophylactic and therapeutic approaches.

Results. Immunization with RAE-1γMCMV vector expressing SIINFEKL epitope provided protection against melanoma challenge in both, prophylactic and therapeutic setting, resulting in postponed or completely prevented tumor growth. Moreover, RAE-1γ expression by the MCMV vector potentiated the induction of KLRG1-expressing SIINFEKL-specific CD8 T cells with enhanced effector properties, conferring long-lasting protection even against a secondary tumor challenge. Furthermore, administration of the checkpoint therapy resulted in improved anti-tumor protection and higher overall survival. The anti-tumor capacity of RAE-1γMCMV vector was even more pronounced upon immunization of newborn mice, providing life-long protection against tumor challenge.

Conclusion. Altogether, our results clearly show that CMV vector expressing RAE-1γ induces KLRG1-expressing CD8 T cells with enhanced anti-tumor properties and can serve as a powerful platform for development of tumor vaccines aimed to prevent and treat CD8 T cell-sensitive tumors.
NKG2D SETS THRESHOLDS FOR SPECIFIC ACTIVATING RECEPTORS EARLY IN NK CELL-DEVELOPMENT

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Introduction. NKG2D and NCR1 (NKp46 in humans) are both activating receptors expressed on all NK cells early in NK cell development and have important role in the cellular stress-surveillance. ‘Stressed’ cells due to viral infection, oncogenic transformation, metabolic or other reasons up-regulate NKG2D and NCR1 ligands (‘induced self’) 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). This results in appearance of mature hyper-responsive NK cells. Klrk1−/− mice show an enhanced NK cell-mediated resistance to MCMV infection, while they still keep impaired ability to kill NKG2D expressing tumor targets.

Aim. In this research we investigated molecular mechanism underlying observed hyperreactivity and how it influence control of tumors which do not express NKG2D ligands.

Materials and Methods. To investigate how observed hyperreactivity influences tumor control we used two tumor models: γ irradiation induced thymoma and B16 melanoma. Using different functional assays and flow cytometry we analysed function of NK cells. Different genetically modified mice were used to investigate roles of specific receptors and signalling molecules.

Results. NKG2D-deficiency results in specific hyperreactivity through NCR1 and CD16 receptors. This 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−/− mice.

Conclusion. This research shows for the first time that an activating receptor can influence activity of another activating NK receptor, which indicates that there is another layer of NK cell regulation. Early during NK cell development NKG2D/DAP12 axis sets threshold for CD16 and NCR1. Loss of this regulation leads to the specific NK cell hyperreactivity resulting in better control of MCMV infection and tumors which do not express NKG2D ligands.
NK CELLS-DERIVED INTERFERON γ MEDIATE MICROGLIA POLARIZATION AND DELAY IN CEREBELLAR GROWTH DURING CONGENITAL CMV INFECTION

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Introduction. Congenital human cytomegalovirus (HCMV) infection is the most common viral cause of long-term neurodevelopmental sequelae, including mental retardation, microcephaly and sensorineural hearing loss. This long-term sequelae seems to be due to proinflammatory response in the brain induced by virus infection. However, the mechanisms and cell types involved in the process are still not elucidated. As HCMV does not cross species barrier, we employed a well-established animal model of congenital CMV infection in which newborn mice are infected with mouse cytomegalovirus (MCMV).

Aim. The aim of this work was to study innate immune responses in the brain and its contribution to delay in cerebellar growth during congenital CMV infection.

Materials and methods. To address this issue, we used MCMV infected newborn mice and followed the kinetics of NK cells infiltration in the brain and impact of infection on the maturation and functional properties of brain infiltrating NK cells. We also followed the kinetics and phenotype of microglia.

Results. We demonstrated that MCMV infection of the newborn mice brain leads to infiltration of NK cells, strong inflammatory response and polarization of microglia towards proinflammatory phenotype. The number of NK cells in the CNS peaked at day 8 post infection (p.i.). Phenotypic analysis showed that brain infiltrating NK cells are highly activated and that they produce IFNγ. NK cells-derived IFNγ appear to be essential in polarization of microglia towards proinflammatory phenotype as depletion of NK cells or neutralization of IFNγ abolished polarization of microglia. Notably, IFNγ neutralization also normalized altered cerebellar development.

Conclusion. These results indicate that IFNγ is a major component of the inflammatory response that is associated with altered neurodevelopment that follows CMV infection and that NK cells that infiltrate the brain represent an effector cell population of IFNγ induced inflammation in this model.
VIRUS-INDUCED IFNγ CAUSES INSULIN RESISTANCE IN MUSCLE AND DERAIDS GLYCEMIC CONTROL IN OBESITY

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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 (6 weeks after start of high fat diet - HFD). 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). 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 respiratory infection increases systemic IR (HOMA-IR) and fasting insulin levels in normal and to more extent in overweight 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 normal weight patients, while in overweight group they remained relatively high. 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 selective insulin resistance in skeletal muscle drives hyperinsulinemia to boost anti-viral immune response and derails glycemic control in obesity, causing rapid progression to type 2 diabetes mellitus.
THE ROLE OF INNATE IMMUNE CELLS IN DEVELOPMENT OF NAFLD

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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 that has a relatively benign prognosis, to steatohepatitis (NASH) and cirrhosis that lead 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 innate immune cells and their receptors that are known as sensors of early changes and are able to induce inflammation.

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 period by FACS. By using different knock-out mouse strains we tried to pinpoint the role of each cell subset and their receptors in development of NASH.

Results. In C57B6J mice first 4 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 and change of their phenotype. They mostly produced pro-inflammatory IL-17A cytokine.

Histological examination of hematoxylin and eosin- (HE) and Sirius red-stained liver sections revealed the development of all stages of NAFLD (steatosis at week 8, severe steatosis with inflammatory changes at week 12 and fibrosis at week 16) in mice that were on SSD diet. In comparison to C57B6J control, TCRδ−/− and TCRα−/− knock out mice had less pronounced hepatic inflammation and fibrosis when fed with SSD diet due to the absence of γδ and αβ T cells respectively. Furthermore, lack of some of NK cell activating receptors, like NCR1, in knock out mice also resulted in decrease of inflammation and fibrosis.

Conclusion. Even though both NK and γδ T cells are parts of innate immunity and they might be the first cells that sense obesity-induced cellular stress in liver, γδ T cells were the only cells that changed their phenotype and started to produce IL-17A.

Until recently, majority of research emphasized the role of αβ T cells in development of fibrosis but we are the first to show that not only them, but also γδ T cells appear to be essential in development of liver inflammation and fibrosis after exposure to SSD.
MOLECULE 1, SELECTIVELY EXPRESSED IN M2-LIKE MACROPHAGES AFFECTING MONOCYTES MOTILITY

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Introduction. Tumor-associated macrophages (TAMs) are key orchestrators of the tumor microenvironment directly affecting many biological activities such as neoplastic cell growth, neoangiogenesis, and extracellular matrix remodelling in order to promote tumor growth. Levels of TAMs often correlated with bad prognosis and more recent studies have also highlighted a link between their abundance and the process of metastasis. Recently, it was shown by gene profiling that human Molecule 1 (Mol-1) is selectively expressed by M2 macrophages and by tumor-associated M2-like macrophages. This observation suggests that Mol-1 could represent a novel marker of macrophage polarization and could exert functional properties in tumor progression.

Materials and Methods. We focus our attention on the characterization of reagents to study Mol-1 biology. After immunization with the Mol-1, we selected a monoclonal antibody recognizing specifically human Mol-1. The antibody was used to purify recombinant human Mol-1 by immunoaffinity chromatography. Initial efforts were aimed at defining the conditions ensuring protein stability over the time. Given that the alternative phenotype can be induced in macrophages by different stimuli, Mol-1 expression was validated upon several anti-inflammatory treatments. The anti-human Mol-1 mAb was used in immunohistology and immunofluorescence to detect the endogenous Mol-1 in sections of paraffin embedded different tumor and normal tissues. In addition to assess Mol-1 mRNA expression was done in the same specimens. In order to assess Mol-1 expressions in tumour associated macrophages (TAMs) following markers were used: CD68 or CD206 or CD163, respectively. In addition, the biological activity of purified rhMol-1 was tested in migration and invasion assays, using boyden chamber or transwells respectively.

Results. An heterogeneous pattern of Mol-1 expression were observed in the tumor tissues. Mostly Mol-1 is found in tumor cells, some cells randomly distributed in stroma, fibroblasts and macrophages. Interestingly, in the double-stained specimens, most CD68 or CD206 or CD163 positive cells were found to express Mol-1. Mol-1 mRNA and protein analysis clearly demonstrated that it is specifically associated with the M2 polarization status of macrophages. Mol-1 can affect the motility of a different human cancer cell line, as well as migration of human monocytes and neutrophils, suggesting that it can promote monocyte/macrophage and neutrophils recruitment into tissues.

Conclusion. Mol-1 is selectively expressed in M2 macrophages and TAMs suggest that could represent a novel marker of macrophage polarization. In addition, modulating monocyte migration, Mol-1 might promote and/or sustain a permissive microenvironment for cancer cell invasion and metastasis. Definitely, further efforts are required to evaluate the prognostic/diagnostic potency of this protein as a M2 marker.
CYTOMEGALOVIRUS-ENCODED PROTEIN M154 AFFECTS AN IMMUNOLOGICALLY RELEVANT LIGAND CD155

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Introduction. Cytomegaloviruses (CMVs) are known for their regulation of immunologically relevant ligands in order to circumvent host immune surveillance. Both human and murine CMV (MCMV) downregulate the surface expression of CD155 (Poliovirus receptor; PVR) molecule, which serves as a ligand for activating receptor CD226 (DNAX Accessory Molecule-1; DNAM-1), but also for inhibitory receptor TIGIT (T-cell Immunoglobulin and ITIM Domain), both present on natural killer (NK) cells. We have previously characterized MCMV-encoded protein, m20.1, as responsible for PVR intracellular retention in an immature form and its commitment to proteolytic degradation leading to a decrease in its surface expression.

Aim. Taking into account the extent of PVR surface downregulation in wild-type MCMV infection, we searched for additional viral-encoded products that interfere with PVR expression.

Materials and methods. Mouse embryonic fibroblasts (MEF), B12, IC-21 and DC2.4 cell lines were infected or not with MCMV WT or MCMV mutants at 1-3 PFU/cell. The expression of PVR, AP-1 (Adaptor protein-1) and viral protein m154 was revealed by flow cytometry and immunofluorescence 16-28 h p.i. PVR gene expression was analyzed by RTqPCR. Balb/c mice w/o NK and CD8+ T cells depletion were infected i.v. with 2 x 10^5 PFU/mouse of MCMVΔm154 or the control MCMV WT. At 3, 4 and 14 d p.i. viral titers in organs were determined by standard plaque assay.

Results. By screening a panel of MCMV deletion mutants, we identified another MCMV product, m154, as a second regulator of PVR. It has been shown previously that m154 participates in immune evasion by inducing the proteasomal and lysosomal degradation of CD48, a high-affinity ligand for NK and cytotoxic T cell receptor CD244. In the case of PVR, our preliminary results suggest that MCMV infection enhanced PVR intracellular accumulation from the cell surface. Intracellularly accumulated PVR colocalized with AP-1 transport vesicles, as well as m154, suggesting the potential involvement of this viral product in the miss-sorting of its target. On the other hand, MCMV infection highly upregulated the PVR transcriptional profile, but m154 is not involved in this modulation. Lastly, MCMVΔm154 is attenuated in vivo and this attenuation is NK cell-dependent early after infection.

Conclusion. We are currently deciphering the exact mechanism of action of m154 on the modulation of PVR, which leads to a decrease of its interaction with NK cell receptors. Taken together, our findings suggest that m154 has a prominent role in viral escape from the host immune surveillance.
CHEMOKINE SIGNALS ARE CRUCIAL FOR ENHANCED HOMING AND DIFFERENTIATION OF CIRCULATING OSTEOCLAST PROGENITOR CELLS

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Background. Peripheral blood (PB) monocyte pool contains osteoclast progenitors (OCPs) which contribute to osteoresorption in inflammatory arthritides, and are influenced by cytokine and chemokine milieu. Our study aimed to define importance of chemokine signals for migration and activation of OCPs in rheumatoid arthritis (RA) and psoriatic arthritis (PsA).

Methods. PB and, when applicable, synovial fluid (SF) samples were collected from 129 RA, 53 PsA and 110 control patients in parallel to clinical parameters of disease activity, autoantibody levels and applied therapy. Receptors for osteoclastogenic factors [CD115 and receptor activator of nuclear factor-κB (RANK)] and selected chemokines (CCR1, CCR2, CCR4, CXCR3, CXCR4) were determined in OCP-rich subpopulation (CD3-CD19-CD56-CD11b+CD14+) by flow cytometry. In parallel, levels of CCL2, CCL3, CCL4, CCL5, CXCL9, CXCL10 and CXCL12 were measured using cytometric bead array or ELISA. Sorted OCPs were stimulated in culture by macrophage colony-stimulating factor and RANK-ligand, and differentiated into mature osteoclasts that resorb bone. Selected chemokines (CCL2, CCL5, CXCL10 and CXCL12) were tested for their osteoclastogenic and chemotactic effects on circulatory OCPs in vitro.

Results. OCP population was moderately enlarged among PB cells in RA and correlated with levels of TNF-α, rheumatoid factor, CCL2 and CCL5. Compared to PB, RANK+ subpopulation was expanded in SF and correlated with the number of tender joints. PsA patients could be distinguished by increased RANK expression rather than total OCP population. OCPs from arthritic patients had higher expression of CCR1, CCR2, CCR4, CXCR3 and CXCR4. In parallel, RA patients had increased levels of CCL2, CCL3, CCL4, CCL5, CXCL9 and CXCL10, with significant elevation in SF vs PB for CXCL10. Subset expressing CXCR4 positively correlated with TNF-α, bone resorption marker and rheumatoid factor, and was reduced in patients treated with DMARD. CCR4+ subset showed significant negative trend during anti-TNF treatment. CCL2, CCL5 and CXCL10 had similar osteoclastogenic effects, with CCL5 showing greatest chemotactic action on OCPs.

Conclusions. Our study identifies distinct effects of selected chemokines on stimulation of OCP mobilization, tissue homing and maturation. Novel insights into migratory behaviors and functional properties of circulatory OCPs in response to chemotactic signals could open ways to new therapeutic targets in RA.
HYPERMETHYLATION OF NLRP3 PROMOTER REGION COULD BE RESPONSIBLE FOR DECREASED GENE EXPRESSION, INFLAMMASOME MALFUNCTION AND GUT DYSBIOSIS IN EARLY PHASE JUVENILE SPONDYLOARTHRITIS

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Introduction. Juvenile spondyloarthritis (jSpA) is a complex disease with both genetic and environmental factors contributing to the etiology. Recently obtained gene signatures in jSpA patients revealed TLR4 and CXCR4 gene had increased, while NLRP3 and PTPN12 had decreased expression, though the mechanism(s) responsible for those alterations remained unknown. To elucidate the possible role of epigenetic modifications in the regulation of those genes, DNA methylation analysis was performed.

Materials and Methods. DNA was isolated from PBMCs of 19 patients diagnosed with jSpA according to ILAR classification criteria for enthesitis related arthritis (ErA) and seven matched healthy children. None of the jSpA participant had symptoms for more than six months and all were untreated. Methylated DNA Immunoprecipitation (MeDIP) was performed in promotor region of differentially expressed genes (TLR4, CXCR4, NLRP3, PTPN12) using the Magmedip kit. Enrichment in MeDIP fraction was determined by quantitative RT-PCR using the AriaMx. MeDIP results were expressed as fold enrichment of immunoprecipitated DNA for each site.

Results. Statistical analysis revealed significant hypermethylation of promoter sites in NLRP3 gene (p=0.0220). No significant alterations in methylation status were observed in promotor regions of other genes.

Conclusion. Our study indicates the hypermethylation of NLRP3 gene promotor is probably responsible for expression alterations in jSpA patients in the initial phase of the disease. The NLRP3 gene has a crucial role in assembly of NLRP3 inflammasome, innate immune sensor that regulates “danger” response upon various signals. While increased expression of this gene has been found in many autoinflammatory diseases, decreased expression has been associated with IBD in which the microbiota is believed to contribute to the intestinal inflammation. Therefore, it is not entirely surprising decreased expression has also been observed in jSpA, the disease that has clinical and genetic similarities with IBD and is often characterized by subclinical gut inflammation. The growing number of evidence shows any modification of gut microbiota can lead to dysbiosis with long-term consequences for the whole organism. Specifically, in jSpA this could result in increased influx of TLR4 ligands and increased expression of TLR4 gene, as well as in reduction of commensal bacteria with anti-inflammatory properties, namely bacteria Faecalibacterium prausnitzii that inhibits NF-κB signaling, leading to TNF-α abundancy characteristic for jSpA. Since inflammasomes have been shown to shape the microbiota, it is reasonable to assume these processes can at least partially be explained by reduced NLRP3 expression due to hypermethylation. Therefore, our findings could have important implications in understanding of the disease mechanisms and possible therapeutic options.
Introduction. Alzheimer’s disease (AD) is progressive neurodegenerative disease and leading cause of cognitive and behaviour impairments of industrialized society. Microbiome-gut-brain axis is bidirectional communication between central and enteric nervous system, thus connecting emotional and cognitive centers in brain with peripheral gut function.

Aim. Aim of the research is to explore changes in the microbiome that happened due to treatment of the rats with aluminium chloride and D-galactose.

Materials and methods. In this research rat model of AD (n=10) was induced by intraperitoneal injection of aluminium chloride (10 mg/kg rat) and D-galactose (60 mg/kg rat) during 28 days. Dilutions were made from rat’s colon content and streaked on selective plates for isolation of Lactobacillus and Bifidobacter, activity of bacterial enzymes was analyzed and DNA isolated for sequencing. Sequencing data was analyzed by hierarchical clustering and principal component analysis.

Results. Results showed: a) reduced number of probiotic bacteria in AD model compared to control group; b) increased activity of β-galactosidase, β-glucosidase i β-glucuronidase in AD model compared to control group c) there was no great changes in composition of intestine microbiome, but 46 bacterial species, which make 11,61% of intestine microbiome of control group, significantly changed.

Conclusion. Based on these results it can be concluded that reduced number of Lactobacillus and Bifidobacter, increased enzyme activity of β-galactosidase, β-glucosidase i β-glucuronidase and reduced intestine biomass are connected with inflammation induced in AD rat model.
THE IMPACT OF HLA-DPB1 MATCHING IN HEMATOPOIETIC STEM CELL TRANSPLANTATION

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Introduction. The impact of patient-donor matching for HLA-A, -B, -C, -DRB1 and -DQB1 genes in hematopoietic stem cell transplantation (HSCT) is well-recognized, but typing for additional genes, such as HLA-DPB1, is still controversial. Based on defined T-cell epitope (TCE) groups, all HLA-DPB1 mismatches can be classified as permissive or non-permissive according to their predicted immunogenicity.

Methods and Materials. We analyzed HLA-DPB1 matching influence on transplantation outcome among patient-matched unrelated donor (MUD) pairs transplanted in 2008-2011 period. HLA typing for HLA-A, -B, -C, -DRB1, -DQB1, and -DPB1 was done using PCR-SSP high resolution method. In this retrospective study we analysed 82 patient-MUD pairs who underwent HSCT, and explored the impact of HLA-DPB1 matches, permissive and non-permissive mismatches on transplantation outcomes.

Results. Patient-MUD pairs matched for HLA-DPB1 alleles in univariate analysis were associated with a significantly higher incidence of disease relapse compared to pairs who were permissive/non-permissive HLA-DPB1 mismatched according to the TCE3 and TCE4 algorithms (P = 0.025 and P = 0.026, respectively), although the significance was lost in multivariate analysis. The analysis did not reveal any significant influence of HLA-DPB1 alleles on overall survival (OS), non-relapse mortality (NRM) or graft-versus-host disease (GvHD) incidence.

Conclusion. In conclusion, our study presents evidence that HLA-DPB1 matching influences the relapse rate in patients after HSCT so the HLA-DPB1 alleles should be implemented in the MUD search algorithm as a transplantation determinant.
POSTER PRESENTATIONS
THE ASSOCIATION OF KIR GENES WITH HEMATOPOIETIC STEM CELL TRANSPLANTATION OUTCOME

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Introduction. Killer cell immunoglobulin-like receptors (KIR) are glycoproteins principally found on the surface of natural killer (NK) cells. The interaction between the KIR receptor and the corresponding ligands, which in most cases are HLA class I molecules, results in the generation of an activating or inhibitory signal in the NK cells and thus regulate their function. In the case of hematopoietic stem cell transplantation (HSCT), KIR receptors of donor cells in the interaction with HLA ligand on the target recipient cells regulate the alloreactivity of NK cells. It has been shown that the presence of donor-derived alloreactive NK cells in the recipient is one potential factor affecting HSCT outcome.

Aim. The aim of this research was to investigate the polymorphism of KIR genes in 55 patients with hematological malignancies who underwent HSCT from matched related donor and determine their possible correlation with diseases and HSCT outcomes.

Materials and Methods. KIR genotyping was performed by the PCR-SSOP method that allows determining the presence or absence of KIR genes and providing information about some allele variants.

Results. The KIR gene frequency analysis showed a significantly higher incidence of full-length KIR2DS4 alleles among patients (P<0.0001). The impact of KIR2DS4 alleles on transplantation outcomes revealed that donors’ full-length KIR2DS4 alleles are associated with lower overall survival rates (P=0.016) and higher relapse incidence (P=0.006).

Conclusion. Our findings suggest that patients transplanted from full-length KIR2DS4 positive donors have worse clinical outcome after HSCT so the KIR typing for KIR2DS4 should be used as an additional criterion for selecting suitable donors in cases when more than one HLA identical donor is identified for a specific patient.
MONOCYTE FUNCTIONS IN BREAST CANCER PATIENTS

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Monocytes and macrophages are involved in the immune reaction and various other physiological tasks through phagocytic function, and the release of cytokines and numerous other soluble molecules. Cooperating with lymphocytes and other cells of the immune response, monocytes take part in the combat against the growth and spread of neoplasms.

The amount of IL-1α and TNF-α released in supernatant cultivate and LPS stimulated monocytes were quantified by radioimmunoassay, further, using acridine orange method, and living yeast cells as targets, we determined ingestion (% of phagocytic yeast) and digestion (% intracellular killing yeast) in 30 patients with breast cancer stage I and II (TNM classification), 1-7 days before and 3-4 weeks after surgery, and also in a group of 40 healthy subjects.

In the peripheral blood of breast cancer patients less monocytes were present; 3% (interquartile range 1-4; Mann-Whitney U test: p=0,012) or 142x10⁶/L 47-248 IR; Mann-Whitney U test: p=0,010). The release of IL-1α was enhanced; 2,14ng/mL (0,93-5,41 IR; Mann-Whitney U test: p=0,010). The release of TNF-α, ingestion and digestion were found to be close to control values. Monocyte number, TNF-α release, and phagocytic function in patients with tumor surgically eliminated is similar to control values, while IL-1α is enhanced 1,10ng/mL (0,80-2,72 IR; Mann-Whitney U test: p=0,002)

More disturbed monocyte functions in patients with tumor compared to patients with eliminated tumor suggests that tumor release soluble products that can effect monocyte functions. Following the phagocytic function of monocytes, ingestion and digestion, which are regulated by TNF-α and IL-1α autocrine, we have an additional possibility to judge different aspects of monocyte activity.
Introduction. Metabolic phenotype of immune cells affects their differentiation and function. Our previous study demonstrated that AICAR (5-amino-1-β-D-ribofuranosyl-imidazole-4-carboxamide), a modulator of AMP-activated protein kinase (AMPK), induced the expression of markers associated with mature monocytes and macrophages, and no such effects were observed in U937 cells treated with metformin, although both AMPK-modulators had similar effects on proliferation and survival.

Aim. Present studies are aimed to determine the mechanism of AICAR-mediated effects in acute myeloid leukemia (AML) cell lines and to test for the role of autophagy and metabolic changes during differentiation of AML cells.

Materials and methods. U937 cells were incubated in the presence of AICAR, A76996, metformin, all-trans retinoic acid (ATRA), phorbol 12-myristate 13-acetate (PMA), bafilomycin and 3-methyladenine (3-MA). The number of viable cells was determined by hemocytometer and the number of colony-forming units by methylcellulose assay (Methocult H4434). The expression of differentiation markers and cell viability were analyzed by flow cytometry (FACSCalibur, Becton Dickinson). Cells transfected with mRFP-GFP-LC3B (Addgene) were analyzed by confocal microscopy (LSM 510, Zeiss). Gene knockdown was performed using siRNA transfections (Neon transfection system, Invitrogen). Total cell lysates were analyzed for the level of LC3B, Beclin-1, PI3KC3, Atg7 and actin by Western blot. Glucose and lactate concentrations in the supernatant were measured using Cobas C5001 (Roche). The data are shown as means±S.E.M. and analyzed using Student t-test or ANOVA.

Results. AICAR increased the level of Thr-phosphorylated AMPK, but siRNA-mediated decrease in AMPK expression had no significant effects on the AICAR-mediated effects on the number of viable cells or the expression of differentiation markers. AICAR-mediated effects were not mimicked by A769662, a more specific direct AMPK-activator. AICAR, all-trans retinoic acid (ATRA) and phorbol esters (PMA) induced autophagy flux, as measured by the level of LC3II in the presence and absence of bafilomycin A, and no increase in the level of autophagy was observed in the presence of metformin. Different effects of AICAR and metformin on autophagy flux were verified by confocal microscopy of mRFP-GFP-LC3B-transfected cells. The autophagy inhibitor 3-methyladenine inhibited differentiation in response to all inducers. The effects of AICAR and ATRA on differentiation markers did not depend on PI3KC3 and Atg7. Preliminary data suggest that ATRA decreases, metformin increases, and AICAR has no significant effects on glucose consumption and lactate production in U937 cells.

Conclusion. These data suggest that aerobic glycolysis and autophagy flux are both associated with differentiation of U937 cells.
COLOCALIZATION OF CYTOTOXIC DYNAMIC DUO – GRANULYSIN AND PERFORIN OF DECIDUAL LYMPHOCYTES, AND THEIR INFLUENCE ON DIFFERENT CELL DEATH TYPES DURING CYTOTOXICITY ASSAY

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Introduction. Early pregnancy decidual lymphocytes are mostly consisted of NK cells. Specific for these NK cells is their phenotype – CD56\(^{\text{bright+}}\) CD16-negative as they possess distinct immunoregulatory role. Granulysin (GNLY) is an important cytotoxic/apoptotic molecule of human NK cells and T cells. Shorter GNLY form (9 kDa) possesses exclusive cytotoxic property, while longer (15 kDa) acts as an “alarmin” and enhances immunological reactions. Perforin (PER) is a cytolytic molecule, and a cofactor of GNLY entry in the eukaryotic cells. They often share the same granules of cytotoxic cells (colocalize). The expression of GNLY and PER in decidua of human early pregnancy is higher than any other condition known. More than 85% of decidual CD56\(^{\text{bright+}}\) NK cells express GNLY and PER.

Aim. We investigated the colocalization of GNLY forms and PER with a marker of degranulation – molecule LAMP-1 in DLs and peripheral blood lymphocytes (PBLs), as well as the influence of proinflammatory cytokines IL-15 and IL-2, and HSP70 on GNLY forms and PER colocalization. Another point of interest was to determine the occurrence of cell death types – early apoptosis, late apoptosis and necrosis mediated by DLs during the cytotoxicity assay, and by blocking with specific GNLY form and PER antibodies to elucidate the role of these mediators.

Materials and methods. Decidual tissues and peripheral blood were obtained from the Clinics of Gynecology and Obstetrics, Clinical Hospital Rijeka after informed consent. The expression of GNLY forms and PER was determined by immunofluorescence and confocal microscopy. Cytotoxicity assay was performed by flow cytometry, and PKH-26-positive K-652 target cell line

Results. IL-15, but not IL-2 in DLs decreased the colocalization of cytotoxic 9 kDa GNLY form with LAMP-1. In DLs stimulated by HSP70 the colocalization both GNLY forms and PER with LAMP-1 marker is higher compared to unstimulated DLs, while in PBLs only cytotoxic form of GNLY and PER colocalize more with LAMP-1. After 5 min there is no influence of anti-GNLY and anti-Per antibodies on apoptosis and necrosis of target cell, but after 18 hours these antibodies decreased the necrosis of target cells.

Conclusion. This unresponsiveness on stimulation with IL-15 could indicate a „cytotoxic inertness” of DLs in using 9 kDa GNLY compared to PBLs. HSP70 molecule at the maternal-embryonic interface could present a threat for embryo. Blocking by anti-GNLY and anti-PER antibodies decreased the necrosis only, possibly due to the inability of method to distinguish apoptosis and necrosis after 18 hours of cultivation.

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CHEMOKINE RECEPTOR EXPRESSION AND MIGRATION OF EXPANDED MYELOID LINEAGE CELLS AND OSTEOCLAST PROGENITOR SUBPOPULATION IN MOUSE MODEL OF RHEUMATOID ARTHRITIS

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Introduction. Overactive osteoclasts, specialized bone resorbing cells, cause bone destruction in rheumatoid arthritis (RA). Osteoclast progenitors (OCPs) arise from myeloid precursors of monocyte/macrophage lineage (ML) and are normally present in the bone marrow and among circulatory monocytes. Under inflammatory conditions in RA their subpopulations possibly go through various changes and get attracted to inflamed sites by yet unknown mechanisms and chemotactic signals.

Objectives. We investigated frequencies, chemokine receptor expression and migration potential of ML cells and OCPs in circulation and periarticular bone marrow of affected joints (PBM) responsible for increased bone resorption in mice with collagen-induced arthritis (CIA), a mouse RA model.

Methods. After receiving Ethical approval, C57BL/6 mice were immunized with chicken type II collagen to induce CIA. Hind paw joints were assessed by micro-CT and histology. Peripheral blood (BL) and distal tibia bone marrow (PBM) cells were analyzed by flow-cytometry for hematopoietic markers and chemokine receptor expression (CD3, B220, NK1.1, CD11b, CD115, CX3CR1, CCR2, CCR5, CCR9, CXCR4). Serum cytokines were measured by ELISA. OCPs were sorted using FACS, cultured with M-CSF and RANKL, stained for TRAP enzyme and counted. For in vitro migration assay, M-CSF and RANKL stimulated PBM and BL cells were seeded into transwell inserts with chemotactic gradient. Intravascular in vivo staining was used to label BL cells for migration tracking.

Results. We found both lymphoid-negative CD45+CD11b+CD115+ and lymphoid-negative CD45+CD11b−/loCD115+ subsets of OCPs to be increased in blood and PBM in CIA. These subsets possess osteoclastogenic activity, express CCR2 and CX3CR1 substantially, and have variable expression of CCR5, CCR9 and CXCR4. CCL2 serum levels are significantly increased in CIA. BL cells from CIA mice show significantly enhanced migration toward CCL2 and CCL5 chemotactic gradient. CD45+CD11b+ cells show increased recirculation through PBM of CIA mice.

Conclusions. ML and OCPs are highly induced in CIA, with substantial expression of CCR2 and CX3CR1, possibly responsible for their increased homing to bone surfaces of inflamed joints. These results suggest that therapeutic blocking of chemokine signaling may be a promising approach to reduce osteoclast activity in RA.
RANKL/RANK/OPG AXIS IS DEREGULATED IN CEREBROSPINAL FLUID OF MULTIPLE SCLEROSIS PATIENTS AT CLINICAL ONSET

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**Introduction.** Multiple sclerosis (MS) is an autoimmune disease of the central nervous system causing neurologic impairment in young adults.

**Objectives.** We hypothesized that MS patients have enhanced RANKL (receptor activator of nuclear factor-κB ligand)/RANK activation as a part of underlying autoimmune disturbance. Our study focused on RANKL/RANK/osteoprotegerin (OPG) axis and selected proinflammatory/immunoregulatory upstream mediators in peripheral blood (PBL) and cerebrospinal fluid (CSF) of MS patients at disease onset compared to healthy control subjects and patients with advanced relapse-remitting form (RR-MS).

**Methods.** PBL and CSF were collected from healthy controls (n=35), MS patients at clinical onset (n=33) and patients with advanced RR-MS (n=30). Patients were assessed by the expanded disability status scale (EDSS) and routine laboratory parameters. Soluble (s)RANKL and OPG were measured in CSF and plasma by ELISA. Gene expression in PBL mononuclear cells was detected by quantitative PCR for RANKL, RANK, OPG and selected cytokines/chemokines (IL-4, IL-10, IL-17, CCL2 and CXCL12).

**Results.** OPG level in CSF was lower in MS at clinical onset, with the significant ability to discriminate between MS patients and healthy controls. sRANKL/OPG ratio was higher in CSF of MS patients at clinical onset and in plasma of RR-MM patients compared to controls. Gene expression of RANKL/RANK/OPG was higher only in RR-MS. IL-4, CCL2 and CXCL12 were in significant positive, whereas IL-10 was in significant negative correlation with RANKL and RANK expression. OPG was in negative correlation with EDSS and alkaline phosphatase level.

**Conclusion.** Our study revealed that RANKL/RANK/OPG axis is involved in the pathogenesis and progression of MS. Therefore, these factors may serve as disease biomarkers and molecular targets of novel therapeutic approaches.
A NOVEL METHOD FOR PRECISE QUANTIFICATION OF IgG IN HYPERIMMUNE HORSE PLASMA

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The hyperimmune horse plasma (HHP), prepared by an active immunisation of horses with an antigen of interest, is a starting material for antitoxin production (tetanus antitoxin, rabies antitoxin, antivenom). Antitoxins are pure horse immunoglobulins or their fragments, with the ability to neutralise respective antigen (pathogen, toxin, venom). Precise determination of the IgG quantity in the starting plasma is a prerequisite for accurate estimation of process efficiency by *in vitro* methods. The currently used methods are SDS-PAGE with the densitometric analysis of electropherograms or the gel filtration chromatography with peak area analysis.

However, these methods are not accurate so methods that measure antibody activity have to be used for precise production process yields monitoring. In the case of antivenoms, these methods are *in vivo* methods in mice.

In order to completely avoid the usage of animals in yield calculation during antivenom downstream processing, we aimed to develop *in vitro* method for precise and accurate measurement of IgG content in HHP.

The principle of our approach was to measure IgG population specific for venom in the HHP, that would serve as a predictor of the whole IgG population. An ELISA was developed in which affinity-purified IgGs from venom-specific HHP served as a standard. The IgG fraction of a plasma is a heterogenous population of immunoglobulins that differ in class, subclass, or specificity. In addition, each plasma sample has a different distribution of these different immunoglobulin molecules. Any change in class or subclass distribution in a sample can affect the ELISA measurement, in which detection is made by a polyclonal anti-horse IgG reagent. So we introduced an individual internal reference for each particular plasma pool measurement. It is prepared by a fast, cheap and 100% efficient protocol (based on the caprylic acid precipitation of non-Ig proteins) for isolation of whole IgG population from the particular plasma pool. The isolation procedure does not change either IgG specificity or class distribution, as we proved by *in vivo* potency/IgG ratio assessment that showed equal values for plasma and pure IgG sample. Such an individual internal IgG reference sample for each plasma was more than 90% pure as determined by gel filtration HPLC and it was possible to precisely measure its IgG concentration (by total protein determination). Such well-characterised IgG sample from each plasma pool served as an internal reference in ELISA, for correction of ELISA results of the respective plasma pool. The newly developed method was validated and proven to fulfill regulatory requirements of pharmaceutical industry for precision, accuracy, linearity and specificity.
THE EFFECTS OF BLENDED MEDICINAL MUSHROOM PREPARATIONS ON SURVIVAL AND IMMUNOLOGICAL AND ANTIMETASTATIC PARAMETERS IN MOUSE CT26.WT COLON CANCER

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Introduction. Medicinal mushrooms are for millennia widely appreciated as natural sources of bioactive substances and are recognized as significant immunomodulatory and antitumor agents. In the last 30 years there has been an increased interest in the use of fungi in (traditional) medicine as well as in determining their therapeutic and medicinal properties, resulting in exponential growth of the number of scientific research papers on their impact on animal and human organism.

Aim. The aim of this paper is to describe the effects of two commercial preparations from medicinal mushrooms, tableted preparation AGARIKON.1 and liquid preparation AGARIKON PLUS (manufacturer: Dr Myko San – Health from Mushrooms) on various tumor parameters with or without 5-fluorouracil. Besides observed effects on a) survival, we also record the effects of mushroom preparations with or without cytostatic drug on b) tumor growth, c) angiogenesis and tumor invasiveness and d) immunological parameters (macrophage function and cytokine expression.

Materials and methods. The study was conducted on Balb/c mice, 2-3 months old, average weight 20-25 grams. The first set of eight groups of animals (control and 7 various combinations of medicinal mushroom preparations and 5-FU) were inoculated with CT26.WT tumor cells and, after a period of 2 weeks, treated with the aforementioned substances for a period of 2 weeks. The survival was observed for a period of 55 days from tumor inoculation, and results were presented using Kaplan-Meier estimator. The second set of eight groups of animals were treated as above, but sacrificed immediately after the end of the treatment period. MMP-2, MMP-9 ELISA tests were performed for the analysis of antimetastatic effects, Th1/Th2/Th17 cytokine panels (ELISA), NO (Griess reagent) and arginase for the analysis of the immunomodulatory effects. Angiogenesis was analyzed through establishing VEGF concentration (ELISA). Additionally, two preventive groups were used in which two mushroom preparations were administered for a week before and after tumor inoculation, and their survival was observed until day 45. after tumor inoculation.

Results. Results show highly statistically significant increases in overall survival rate and life span in treated groups, as well as significant changes in various immunological and antimetastatic parameters.

Conclusion. The results show significant beneficial effects on various parameters related to tumor suppression and show that medicinal mushrooms preparations alone and in combination with standard therapies have a major role in this in vivo model of colorectal cancer, which should be further explored in the clinic.
CHARACTERIZATION OF NEURODEGENERATION IN AN OPTINEURIN INSUFFICIENCY MOUSE MODEL

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Introduction. Optineurin is a multifunctional poly-ubiquitin (Ub)-binding adaptor protein implicated in several vital cellular functions, such as cell signaling, stress response, autophagy, and vesicular trafficking. Recently, various optineurin mutations have been found in patients with amyotrophic lateral sclerosis (ALS). ALS is a rapidly progressive fatal neurodegeneration. Histopathologically it is characterized by upper and lower motor neuron degeneration, which consequently leads to retrograde axonal loss, and is accompanied by neuroinflammation. Affected neurons contain intracellular inclusions, which are commonly ubiquinated and in >98% of cases contain aggregated TDP-43.

Aim. To assess the potential role of optineurin in neurodegeneration, we designed an optineurin insufficiency mouse model (Optn⁴⁷⁰T) that lacks the C-terminus harbouring the Ub-binding region.

Materials and Methods. The immunohistochemical analysis was conducted on homozygous Optn⁴⁷⁰T mice and the age matched wild type animals. Primary microglia was analysed for signs of impaired inflammatory signalling upon in vitro culture.

Results. In the motor cortex of Optn⁴⁷⁰T mice we found more extensive GFAP immunopositivity and slightly lower density of NeuN positive cells compared to the same brain regions of wild type animals. In mutated animals, a higher proportion of cortical cells showed nuclear NF-κB translocation. In addition, sporadic nuclear 8-oxoguanine immunopositivity was present in Optn⁴⁷⁰T mice and not in their wild type counterparts. In Optn⁴⁷⁰T mice neurofilament H staining was localized predominantly in the neuronal soma, while diffuse axonal immunopositivity was found in control animals. Most importantly, ubiquitinated TDP-43-positive inclusions were much more prevalent in the frontal cortices of the aging Optn⁴⁷⁰T mice. Our in vitro studies with primary microglia showed that optineurin and/or its Ub-binding is dispensable for (acute) NF-κB activation upon Toll-like receptor (TLR) stimulation with LPS. In contrast, LPS and poly-I:C stimulated Optn⁴⁷⁰T microglia had diminished Tank-binding kinase 1 (TBK1) activation, interferon regulatory factor 3 (IRF3) phosphorylation, and IFN-β production, arguing that optineurin is a positive regulator of type I IFN signalling pathway. Several genes distal from IFN-β signalling were also diminished in optineurin Optn⁴⁷⁰T microglia, such as IRF7, RANTES, CXCL10 and NOS2.

Conclusion. In optineurin insufficient mice we observed pathohistological hallmarks of neurodegenerative processes as well as the disbalance of proinflammatory and anti-inflammatory factors in primary microglia upon culture. Together these results argue for the neuroprotective role of optineurin.
RAE-1γΔMAT – A POTENTIAL NOVEL MCMV VACCINE VECTOR

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Introduction. Human cytomegalovirus (HCMV) is a pathogen that causes a lifelong infection which can lead to a life-threatening disease in patients with immature or compromised immune system. A widely used model for studying the mechanism of HCMV infection is the infection of mice with murine cytomegalovirus (MCMV), due to their genetic, biological and pathogenic similarities. We have previously identified a novel highly abundant MCMV transcript MAT that encodes 2 proteins. One of MAT-encoded proteins regulates surface amount of MHC I molecules in addition to known MCMV MHC I modulator m04. Removal of this protein results in lesser amount of MHC I on the surface of the infected cells and renders the cell susceptible to NK cell-mediated “missing-self” recognition. In addition, removal of MAT also resulted in the qualitative and quantitative properties of MHC I-presented peptides. These characteristics of MAT might be of use in the development of live attenuated CMV vaccine vector that is another topic of interest in our laboratory.

Aim. Several characteristics make CMV a good vaccine-vector candidate: CMV can induce strong and durable CD8+ T cell response, its large genome can be manipulated to contain several transgenes and it possesses numerous immunoevasion genes whose removal from the genome render the virus susceptible to immune control. Our laboratory has previously constructed a recombinant MCMV-vector expressing ligand for NKG2D receptor that showed excellent vaccine-vector properties against viral and bacterial infections and tumors - RAE-1γMCMV. We have thus reasoned that removal of MAT, as a regulator of MHC I-presented peptides, might prove beneficial in the context of our already well-established RAE-1γMCMV vaccine vector - RAE-1γΔMAT-MCMV.

Materials and methods. We constructed a new mutant virus RAE-1γΔMAT-MCMV and performed in vivo characterization of its growth by infecting Balb/c mice and analysing virus titers in different organs by plaque assay. Also, we performed IFNγ secretion assay to test the ability of RAE-1γΔMAT-MCMV to induce CD8+ T cell response in infected mice.

Results. In a series of experiments we demonstrated that RAE-1γΔMAT-MCMV is highly attenuated in all tested organs 3, 7 and 28 days post infection. However, we could not observe any improved CD8+ T cell responses between RAE-1γMCMV and RAE-1γΔMAT-MCMV.

Conclusion. MCMV-RAE1γΔMAT virus did not show better characteristics compared with Rae1Y-MCMV.
MOLECULAR AND FUNCTIONAL CHARACTERIZATION OF M116 REGION IN MCMV

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Introduction. Human cytomegalovirus (HCMV) is a pathogen that causes a lifelong infection that can lead to a life-threatening disease in patients with immature or compromised immune system. A widely used model for studying the mechanism of HCMV infection is the infection of mice with murine cytomegalovirus (MCMV), due to their genetic, biological and pathological similarities.

Aim. We have previously identified M116 as one of the most extensively transcribed regions of MCMV genome. Despite this, its function is still not known so our goal is to perform molecular and functional characterization of this region and determine the function M116 plays in the pathogenesis of MCMV infection.

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. Molecular characterization revealed at least two 5′ co-terminal transcripts of different sizes in M116 region with the shorter one being spliced. Next, at least one protein encoded by the transcripts of the M116 region was detected. In addition, we showed that it is expressed with late kinetics, it is glycosylated and potentially secreted out of the cell.

In vitro analysis showed comparable growth kinetics of ΔM116 and WT MCMV in primary MEF, however ΔM116 was attenuated in GM-CSF cultured bone marrow cells; BMDCs and BMDMs. Moreover, BMDCs and BMDMs infected with ΔM116 produced more proinflammatory cytokines IL6 and TNFα and expressed more NOS and Arg2, markers of proinflammatory myeloid cells. Interestingly, ΔM116 was not attenuated in vivo.

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.
INTRODUCTION. Phenolic acids are plants polyphenols that are considered beneficial because of their potential protective role in the pathogenesis of multiple diseases associated to oxidative stress such as cancer, cardiovascular and neurodegenerative diseases. Anticancer mechanisms, anti-inflammatory, antioxidant and antiproliferative activities of phenolic acids like caffeic (CA), gallic (GA) and tannic (TA) acids are associated with induction of detoxification enzymes and inhibition of bioactive enzymes. Vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) are proangiogenic factors which play an important role in the invasion, metastasis and angiogenesis of cancer cells. Therefore, VEGF and MMPs are one of the targets for agents to suppress their expression and/or activities and that could inhibit the migration and invasion of cancer cells.

Aim. The aim of this study was to investigate the molecular mechanisms of CA, GA and TA in the inhibition of angiogenesis and growth of Ehrlich ascites tumor (EAT) in mouse.

Materials and methods. EAT cells (2.5 x 10^6) were injected intraperitoneally (i.p.) in Swiss albino mice. One day after tumor inoculation, mice were injected i.p. with caffeic acid (CA) and gallic acid (GA) at dose of 40 and 80 mg/kg bw and tannic acid (TA) at dose of 5 and 10 mg/kg bw during 10 days. On day 14, we have analyzed the ascites volume, the total number of tumor cells in the peritoneal cavity, the microvessel density, the functional activity of macrophages, cyclooxygenase-2 (COX-2), vascular endothelial growth factor (VEGF) and matrix metalloproteinase-2 (MMP-2) and -9 (MMP-9).

Results. CA at dose of 80 mg/kg bw has the highest inhibitory effect on reduced ascites secretion and number of tumor cells. The biggest inhibition of COX-2 activity showed CA 80 mg/kg bw and TA 10 mg/kg bw which correlates with the increased activation of macrophages in ascites fluid, reduced number of blood vessels in peritoneum and decreased activity of MMP-2 in tumor cells. At the same time, TA 5 mg/kg bw significantly reduces level of VEGF in tumor cells. CA 40 mg/kg bw and GA 80 mg/kg bw demonstrated the highest inhibitory effect on activity of MMP-9 in tumor cells.

Conclusion. Caffeic, gallic and tannic acids are able to suppress angiogenesis and tumor growth in Ehrlich ascites tumor and thus they may be a potential chemotherapeutic agents for cancer.
EFFECT OF GRAPE SEED PROANTHOCYANIDINS ON RETINOIC ACID-INDUCED BONE LOSS IN RATS

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Introduction. Osteoporosis is a skeletal disease characterized by the significant decrease in bone mineral density (BMD) and bone microarchitecture disorder. The risk of osteoporosis can be genetic or acquired. Contributing to acquire risk are numerous medications, including 13-cis retinoic acid (13cRA). The main effects of 13cRA that contribute to bone loss are: a) increased oxidative stress (OS) and the formation of reactive radicals, decreased activity of osteoblasts and increased osteoclast activity; b) osteoblasts apoptosis; c) decreased levels of estrogen and appearance of inflammatory cytokines, d) reducing the activity of vitamin D receptor, decrease of the absorption of Ca²⁺ in the intestine, increasing excretion of calcium through the kidneys; d) the effects on parathyroid hormone. Epidemiological evidence has indicated a link between dietary intake of antioxidants such as grape seed proanthocyanidins and bone health.

Aim. The present study investigates the antiosteoporotic effects of grape seed proanthocyanidins (GSPs), natural antioxidants, in a 13cRA-induced osteoporosis model of rats and healthy rats.

Materials and methods. Three month-old female rats of the Y59 strain were given GSPs (100 mg kg⁻¹) or alendronate (AL; 40 mg kg⁻¹, a positive control) concomitant with 13cRA (80 mg kg⁻¹) once daily for 14 days by a single intragastric (i.g.) application. Twenty four hours after the treatment, we analysed bone turnover serum biochemical markers, such as: osteocalcin (OC), C-terminal fragment of type I collagen (CTX), bone mineral density (BMD), bone ash contents, calcium (Ca) and phosphorus (P) content, geometrical and physical parameters of bone as well as oxidative stress parameters (glutathione levels and lipid peroxidation).

Results. 13cRA successfully induced osteoporosis-like changes in rats. The treatment with GSPs increased BMD, OC level, femoral geometric characteristics, Ca and P content (P=0.025; P=0.025) in 13cRA-induced bone loss model. In addition, GSPs-treated rats had significantly lower serum alkaline phosphatase activity (P<0.001) and malondialdehyde (MDA) levels in both liver and kidney (P<0.05). Histological results showed its protective action as well, through promotion of bone formation.

Conclusion. The beneficial role of GSPs against the 13cRA-induced bone loss in rats indicates it could have similar protective action on bone health in humans and be an effective replacement for AL.
SIMPLE AND EFFECTIVE APPROACH FOR ANTIVENOM MANUFACTURING

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Introduction. Antibody-based therapeutics play an important role in specific treatment of some medical emergencies (toxin and viral neutralisation, overcoming of immunoglobulin deficiencies). Their safety, effectiveness and availability are critically dependent on optimised, high yielding and low-cost manufacturing process.

Aim. Here, we report simple, feasible and economically viable purification strategy for preparation of horse plasma-derived antivenom immunoglobulins and/or \( F(ab')_2 \) fragments, that are irreplaceable for counteracting post-snakebite pathophysiological manifestations.

Materials and methods. Fractionation of hyperimmune plasma pool was performed in only few simple and easily scalable purification steps, all designed to thoroughly keep desired IgG molecules or their fragments in solution, preventing possible degradation/aggregation of the active principle due to precipitation or binding to chromatographic supports. Firstly, unwanted plasma proteins, mostly albumin, were precipitated by caprylic acid. IgG-enriched fraction, refined from precipitating agent, was used for pepsin digestion. The final product, \( F(ab')_2 \) fragments, was polished by ion-exchange chromatography on CIM QA disk under conditions which prefer binding of pepsin and by-products of enzymatic digestion exclusively.

Results and conclusion. Developed procedure gives completely pure and aggregates-free \( F(ab')_2 \)-based product of over 75% yield, as monitored by several in vitro methods. In addition, highly pure IgG fraction, an intermediate produced with almost no losses in the described procedure, is also in compliance with regulatory requirements concerning impurity content. So the described technology might serve as a platform for producing horse antitoxins of both types (Ig- and \( F(ab')_2 \)-based) and for other medical purposed, also.
THE INCREASE OF CYTOTOXIC OVER REGULATORY FORM OF GRANULYSIN IN PERIPHERAL BLOOD LYMPHOCYTES OF PATIENTS WITH OSTEOARTHRITIS

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Introduction. Cytotoxic (9 kDa) form of granulysin (GNLY) can induce unscheduled apoptosis of target cell during many pathophysiological processes in human. Its precursor of 15 kDa acts as the enhancer of immune reaction by chemotactic and maturation properties for lymphocytes or dendritic cells, respectively. We have previously shown the increased frequency of GNLY positive cells in peripheral blood lymphocytes of women with knee osteoarthritis (OA) by flow cytometry using anti-GNLY mAb (RC8 clone) which cannot distinguish between cytotoxic and regulatory forms.

Aim. The aim of this study was to investigate the expression of cytotoxic and regulatory forms of GNLY and their intracellular setting in relation of LAMP-1, the marker of exocytotic granules by confocal microscopy.

Material and Methods. Peripheral blood mononuclear cells were isolated by gradient density centrifugation of 17 women with knee OA and 15 healthy women in the control group of the appropriate age after the clinical examination, routine laboratory testing and signing the informed consent. Immunofluorescence was performed using primary polyclonal rabbit Abs recognizing 9 kDa GNLY, mouse RF10 mAb recognizing 15 kDa GNLY, mouse H4A3 clone anti-LAMP-1 or isotype controls. Secondary antibodies: anti-mouse and anti-rabbit mAb were conjugated with green fluorescence Alexa Fluor 488 or red fluorescence Alexa Fluor 594, respectively. Microphotographs were acquired with the Olympus BX51 fluorescence microscope using the Olympus DP71 camera and Cell ^ A program, version 3.0 (Olympus, Tokyo, Japan). Yellow fluorescence appears after the overlaid of green and red fluorescence with Adobe Photoshop, version 7.0.1 CE (Adobe Systems Inc., San Jose, CA, USA).

Results. In a patient with OA, the cytotoxic form of GNLY (9 kDa) was labeled more intensively than regulatory GNLY form of 15 kDa and it was contained in polarized granules. The regulatory form was contained in granules directly below the cell membrane. Cytotoxic form of GNLY colocalized more with LAMP-1 than regulatory form of GNLY.

Conclusion. The increase in the expression of cytotoxic over regulatory GNLY form in peripheral blood lymphocytes suggests the involvement of activated GNLY positive lymphocytes in the pathogenesis of OA at the systemic level.

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OXIDATIVE STRESS AND INFLAMMATION CAUSED BY N-HEXYL SALICYLATE IN MOUSE SKIN: THE EFFECTIVENESS OF FLAVONOIDS

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Introduction. Reactive oxygen species play a role in a number of degenerative conditions including psoriasis. Psoriasis is a chronic inflammatory disease whose etiopathogenesis is not yet completely understood, and therefore there is no standardized therapeutical approach. Flavonoids, recognized as potent antioxidants, are multifunctional molecules that can act as anti-inflammatory and antiproliferative agents through the modulation of multiple signaling pathways.

Aim. The present study was designed to investigate the protective role of flavonoids [quercetin, chrysin, curcumin or Epigallocatechin 3-gallate (EGCG)] in reducing oxidative stress and inflammation in n-Hexyl salicylate (HXS)-induced psoriasis like lesions in skin.

Materials and methods. Psoriasiform lesion was induced by topical application of irritant n-Hexyl salicylate in an area of ~3 cm shaved abdomen of male Swiss albino mice. The seventy mice were divided into 10 groups (each group consisted of 7 mice). The first group were topically treated only with n-Hexyl salicylate and acetone/olive oil (1:1) solution. Next groups (2-5) were treated with irritant Hexyl salicylate and/or Epigallocatechin 3-gallate, quercetin, chrysin, curcumin. Groups 6-9 consisted of mice were treated only with EGCG, QU, CRYS or CURC and control group treated with acetone/olive oil (1:1) solution. During 5 days every animal was topically treated with its specific test compound on the shaved abdomen. Anti-oxidative and anti-inflammatory effect of flavonoids is quantified by histopathological assessment of skin, measuring the levels of lipid peroxidation and glutathione (GSH) in the skin, total number of inflammatory cells in peritoneal cavity, macrophage spreading index, and hematological and biochemical parameters.

Results. Topically applied of n-Hexyl salicylate caused significant increase in lipid peroxidation and decrease in GSH, which is accompanied by an increase total number of inflammatory cells in skin and peritoneal cavity, functional activity of macrophages, and enzymatic activity of ALP and AST. In contrast, topically applied 5 % preparation of flavonoids (quercetin, chrysin, curcumin or EGCG) with HXS effectively prevented these alterations and maintained the antioxidant status.

Conclusion. Results support the use of flavonoids as an anti-inflammatory and antioxidative agent and open up new possibilities for its use in skin disorders. The protective effect of flavonoids against HXS-induced psoriatic lesions is probably based on the reduction of reactive oxygen species, increasing the levels of glutathione and reduced infiltration of macrophages and neutrophils. The protective role of the flavonoids against the inflammation induced by HXS in mice gives hope that flavonoids can control the production of ROS to reduce inflammation of the skin and achieve a similar protective effect in humans.
PROTECTIVE EFFECT OF LIPOPOLYSACCHARIDE-INDUCED INFLAMMATION ON FAS-MEDIATED HEPATOCYTE APOPTOSIS IS MEDIATED THROUGH JAK/STAT3 SIGNALLING PATHWAY

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Introduction. Fas/Fas ligand (FasL) apoptotic pathway is involved in the pathogenesis of various diseases, including liver diseases. However, the exact effects of acute inflammation on the liver apoptotic processes are still not well elucidated. We have previously found that lipopolysaccharide (LPS)-induced acute inflammation alleviates Fas/FasL-mediated apoptosis. Various cytokines (like IL-6, TNF-alpha, IL-1 etc.) and signalling pathways (JAK/Stat3, NF-κB) could mediate this protective effect, but underlying mechanisms are still unknown.

Aim. To explore protective mechanisms and to evaluate if Janus kinase (JAK)/Stat3 signaling pathway plays a pivotal role in the (LPS)-induced protective effect of inflammation.

Methods. Male C57BL/6 mice received intraperitoneal injection of LPS (0.1 µg/g) while the control group of animals received the vehicle (sterile saline). After 2 hours both groups were treated with anti-Fas (JO2) activating antibody (0.25 µg/g, intravenously). Mice were sacrificed after additional 6 hours and plasma (ALT) and liver samples (caspase activity) were harvested. In the second set of experiments, mice were treated with saline or LPS, liver samples (qPCR) and non-parenchymal liver cells were harvested and leukocytes populations were determined by flow cytometric analysis. Concentrations of soluble Fas (sFas) in plasma were determined by ELISA. To explore the possible involvement of JAK/Stat3 pathway, mice were pre-treated with ruxolitinib (JAK-inhibitor, 100 mg/kg, oral gavage) or vehicle (Peg300/DMSO/dH2O), followed by LPS and anti-Fas antibody as before. ALT was measured from plasma samples.

Results. Mice pre-treated with LPS had significantly lower levels of ALT in plasma (median (IQR); 82 (32-182) vs. 3709 (1429-5922) U/L, p=0.02) and lower caspase-8 activity compared to mice which received saline before anti-Fas antibody. LPS treatment increased the expression of TNF-alpha, IL-1 and IL-6 in hematopoietic liver cells, and Fas, CFLAR and Bcl2l1 in hepatocytes. LPS didn’t increase the level of sFas in plasma. Flow cytometric analysis of intrahepatic leukocytes showed an increase in neutrophil (7.6 fold), NK cell (1.7 fold) and NKT cell (1.6 fold) population. Preliminary results showed that ruxolitinib+LPS pre-treated mice had a considerable (but insignificant) increase of ALT levels in plasma compared to mice which received vehicle+LPS prior to anti-Fas treatment (490 (260-2817) vs. 211 (48-439) U/L respectively; p=0.063).

Conclusion. LPS-induced acute inflammation mitigates Fas/FasL-mediated apoptosis by acting on Fas-apoptotic pathway at the level of caspase-8 or upstream of it’s activation. Inhibition of JAK signalling with ruxolitinib reversed this LPS-induced effect considerably, suggesting that Stat3 could be one of the crucial mediators involved in this protective effect.
THE INFLUENCE OF ENZYME MATRIX METALLOPROTEINASE-2 AND 9 AND REGULATORY T CELL IMMUNITY IN THE PATHOGENESIS OF ATHEROSCLEROSIS

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Cardiovascular diseases (atherosclerosis, coronary artery disease, peripheral vascular disease, stroke) are leading causes of morbidity and mortality in all developed and developing countries. Atherosclerosis represents an inflammatory, chronic metabolic disorder of the vessel wall, accompanied with unregular innate and adaptive immune responses, which may cause the progression of the disease. Inflammatory process is stimulated by innate signals and migration of T cells and monocytes in the arterial wall which is crucial for atherogenesis. oxidized low-density lipoprotein (oxLDL) in vitro can contribute to Treg/Th17 balance in the periphery inducing the apoptosis of regulatory T cells (Tregs) and the proliferation of Th17.

The aim of our study was to examine the interactions between the concentration of enzyme matrix metalloproteinases 2 and 9 (MMP-2 and 9) in urine, as well as, the number of Tregs in peripheral blood of patients with mild atherosclerosis (A patients) from the general practice and with carotid arteries stenosis (CAS patients)) who were undergoing the surgical procedure and comparison their results with these in healthy blood donors.

For the investigation of the concentration of enzyme MMPs, we used the method of enzyme immunnoassay (ELISA). Tregs was analysed by flow cytometric method. Our data has showed a large increase in the enzyme MMP- 2 and 9 in the urine of CAS and A patients in comparison with healthy controls, indicated this method as an easy marker for the monitoring of the development of atherosclerosis.

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ANTIOXIDATIVE AND ANTIDIABETIC EFFECTS OF NARINGIN AND CURCUMIN IN VITRO AND IN VIVO

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Introduction. Oxidative stress plays an important role in the etiology of diabetes mellitus and can produce damage on cellular macromolecules, such as DNA and lipids in cellular membrane, impair protein function, and trigger cell death. Many polyphenols, exert positive effects on diseases caused by oxidative stress. Curcumin and naringin differ in number and arrangement of the hydroxyl groups, as well as by the nature and extent of alkylation. They can act as reducing agents, free radical scavengers, metal chelators, and singlet oxygen quenchers. The antioxidant activity of curcumin and naringin in vitro are compared with some of their antidiabetic effect in vivo on molecular cellular and organism level.

Aim. The aim of the present study was to assess whether naringin or curcumin can influence oxidative stress induced DNA damage in mice with alloxan-induced diabetes.

Materials and methods. Diabetes was induced in Swiss albino mice with a single intravenous injection of alloxan at dose of 75 mg kg⁻¹ body weight. Two days after alloxan injection, naringin or curcumin preparations (50 mg kg⁻¹) were given intraperitoneally for 7 days. In order to evaluate the effectiveness of naringin and curcumin we used three assays which cover different aspects of antioxidant activity, observed changes in body weight and survival of mice and used the alkaline comet and micronucleus assays.

Results. In vitro curcumin showed appreciable antioxidant properties, while naringin was much less effective. Naringin or curcumin administration to diabetic mice resulted in decreased DNA damage in lymphocytes and increased level of DNA damage in liver, kidney and reticulocytes. Administration of naringin and curcumin resulted in significant increase of the body weight and 100% survival of mice.

Conclusion. Results suggests that antioxidant activity of naringin and curcumin leads to long time survival of diabetic mice and possible prevention of further oxidative damage, so they could be candidates for antidiabetic agent, but the precise targets of naringin and curcumin in diabetic mice are still to be clarified.
ASSESSMENT OF SYSTEMIC AND LOCAL BONE LOSS IN THE MODEL OF ANTIGEN-INDUCED ARTHRITIS

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**Introduction.** Rheumatoid arthritis (RA) is a chronic autoimmune arthritis characterized by bone and cartilage destruction in the inflamed joints, as well as systemic osteopenia. Systemic and local bone loss involve separate mechanisms and have a different clinical significance. Among various murine models developed and applied in order to study pathogenic mechanisms of RA, antigen-induced arthritis (AIA) is considered to have few or no systemic effects. This model should exclusively mimic the local destructive changes. However, as the immunization protocol induces a systemic immune response we suspected that it may also affect bone mass at the systemic level.

**Aims.** The objective of this study was to determine the extent of local bone destruction and generalized osteopenia in AIA. To achieve that, we assessed the trabecular bone volume in the second lumbar vertebrae (L2), femoral epiphyses and metaphyses, using a micro-computerized tomography. To assess the systemic effect of immunization, we assessed the proportion of myeloid (CD11b+Gr1+) cells in the spleen, femoral bone marrow and knee joints by flow cytometry.

**Materials and methods.** 8-10 week old male C57BL/6 mice were divided into three groups: non-immunized (NI), immunized control (CTRL), and arthritis group (AIA). Immunization protocol in both CTRL and AIA group consisted of two subcutaneous (s.c.) weekly injections of methylated bovine serum albumin (mBSA), followed by intraarticular (i.a.) injection of mBSA (AIA) or phosphate-buffered saline (PBS) (CTRL) two weeks after second injection. NI group was injected with PBS only at all points. Mice were sacrificed 10 days post i.a. injection, when L2, knee joints and spleens were harvested for analysis.

**Results.** Statistically significant decreases in bone volume were found in the L2 and metaphyses of CTRL and AIA mice, in comparison to NI mice (p<0.05, Kruskal-Wallis test). On the other hand, the epiphyseal trabecular bone volume was significantly lower in AIA mice in comparison to ctrl and NI mice (p<0.05, Kruskal-Wallis test). In both immunized groups there was a significant increase in the proportion of myeloid cells in spleen, bone marrow and knee joints, in comparison to the control group.

**Conclusion.** The AIA model of arthritis induces systemic osteopenia, which should be taken into account, in particular when addressing the local osteodestructive changes. Metaphyseal bone volume better reflects the systemic features of the model, whereas epiphyseal trabecular bone volume is a more reliable indicator of the local osteodestructive changes.
THE IMPACT OF NOTCH SIGNALLING ON APOPTOSIS IN CHRONIC LYMPHOCYTIC LEUKAEMIA

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Introduction. Studies of Notch receptors, ligands and downstream targets have challenged the view of leukemogenesis in several diseases as a consequence of impaired microenvironment for lymphocyte differentiation. The role of Notch signalling in promoting survival and apoptosis resistance has been studied in T-ALL, but the efforts implemented regarding this pathway in Chronic lymphocytic leukaemia (CLL) still remains controversial. CLL is one of the most frequent blood cancers in adults in USA, Europe and Australia. The heterogeneous clinical presentation is attributable to several different pathways that contribute to the survival of B-CLL cells. Several groups including our own, contributed to the broadly accepted view of in vivo resistance to apoptosis characterising B-CLL cells. We tackled the question if apoptosis can be influenced or unblocked when the cells are placed in cultures with a lymphocyte supportive microenvironments.

Aim. The aim of this study is to assess the impact of Notch signalling on the survival of leukemic cells in vitro.

Materials and methods. Lymphocytes were separated by centrifugation on a density gradient and co-cultured with activating ligands. mRNA was quantified by real-time RT-PCR. Protein expression was determined by means of flow cytometry.

Results. Notch1 was differentially expressed in B-CLL, both at the transcriptional and protein level. We confirmed our previous results showing an elevated expression of Aiolos. The expression of Notch downstream targets, Deltex1 and Hes1, does not necessarily follow that of Notch1. Hes1 expression was low or non-existent indicating another way of activation through Notch. BCL-2 was present at low levels in T and B cell populations, and increased in leukemic cells, confirming its role in evading cell death. Our co-cultures were established in an optimal system that supports lymphocyte survival. Our results show that the number, both absolute and percentage, of early apoptotic cells increases during the period observed while we found no continuity in the share of late apoptotic cells. The same applies for control cultures without Notch1 stimulation. If the cells were not exposed to DLL1 there was a decrease of cells exhibiting apoptotic traits. The percentage of early and total apoptotic cells is lower than that of cells stimulated by DLL1.

Conclusion. The differential Notch1 expression reflects the heterogeneity of the disease while the expression of its downstream target genes points towards additional mechanisms of regulation. Further research is necessary to resolve these points. Notch signaling analysis in leukemia could contribute to efficient and person-oriented therapy in the future.
HYPOMETHYLATION OF ASC PROMOTER REGION IN TUMOR TISSUE OF PATIENTS WITH NSCLC

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Introduction/aim. DNA methylation, an epigenetic alteration, can affect gene expression without changing the DNA itself. Aberrant methylation present in CpG islands of gene promoter regions can contribute to tumor promotion and is recognized as a hallmark of lung cancer. Many epidemiological studies have linked chronic inflammation to cancer development. Upon insult, cells act together to trigger inflammation via the activation of pattern recognition receptors (PRRs). Subgroups of PPRs, such as NLRs or ALRs, can recognize their ligands and form multiprotein platforms called inflammasomes. Activation of the inflammasome is required for the activation of caspase-1 and the subsequent secretion of proinflammatory cytokines IL-18 and IL-1β. A critical adaptor molecule in inflammasome activation is apoptosis-associated speck-like protein containing a CARD/target of methylation-induced silencing-1 (ASC/TMS1). Since the activation and controlling mechanisms of inflammation are regulated by NF-κB and rely on PRRs, the suppression of these pathways may provide opportunities for prevention and treatment of cancer. The aim of the study was to evaluate the methylation status of the ASC/TMS1 promoter region and to correlate methylation status with its mRNA and protein expression.

Material and Methods. To define the methylation of the ASC/TMS1 gene promoter region in tumor and paired lung tissue the pyrosequencing approach was used. To correlate the methylation status of the promoter region with protein expression, western blotting was performed and the results were quantified by densitometry. The correlation of the hypomethylation of the ASC/TMS1 promoter and the overall survival and tumor grade of NSCLC patients was assessed. To determine the expression of genes involved in TLR and NOD signaling, RT-qPCR was performed.

Results. The promoter region of ASC/TMS1 in tumor tissues in patients with lung cancer exhibited a reduced methylation status compared to healthy tissue. The reduced methylation status of ASC/TMS1 promoter in tumor tissues correlated with higher protein expression and vice versa. In addition, the expression of ASC mRNA in tumor tissues was up-regulated compared to healthy lung tissues. A tumor-specific cytokine profile was found on the mRNA level as well: up-regulation of cytokines such as IL-18 and IL-1β and down-regulation of cytokines such as IL-12A, IL33 and IL-6. According to survival analysis, out of 11 CpGs tested in the ASC/TMS1 promoter region, only hypomethylated CpG4 was found to be associated with decreased overall survival, while higher methylation of CpG8 of the ASC/TMS1 gene was associated with TNM stage 2.

Conclusion. Hypomethylation of the ASC/TMS1 promoter region in tumor tissues of patients with non-small cell lung cancer (NSCLC) contributes to higher ACT/TMS1 mRNA levels and protein expression compared to healthy tissue and is associated with tumor grade and overall survival of patients with NSCLC.
DEVELOPMENTAL ORIGIN OF HEALTH AND DISEASE HYPOTHESIS IN ALLERGIC DISEASES

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Introduction. According to the Developmental origin of health and disease hypothesis, environmental pressure at critical, or early period of development can evoke changes in the gene regulation affecting allergy development. The aim of the study was to explore whether the rural environment of National park Lonjsko polje (NPLP) influences development of allergic sensitisation and disease symptoms in children during schoolgoing ages. Also, we aim to gain in sight into environmental, possibly modifiable factors for reducing risk of allergic disease development.

Methods. Original ISAAC (International Study of Allergy and Asthma in Childhood) questionnaires, consisting of questions on child’s demographic characteristics, core modules on wheezing, atopic dermatitis and allergic rhinitis and supplementary modules on risk factors (smoking, number of children in families, perinatal and family history, home and living environment, nutrition aspects, vaccination, medicines) were completed by parents of 6-7 (6 years 0 months - 6 years 11 months), 10-11 (10 years 0 months - 10 years 11 months) and 13-14 year-old (13 years 0 months - 13 years 11 months) children from five elementary schools of the surrounding area of The Natural Park Lonjsko Polje. A total number of 450 questionnaires were returned and analysed.

Preliminary results. 12- months prevalence of allergic disease symptoms: Age group 6 years 0 months- 6 years 11 months: Self-reported prevalence of wheezing (Wh) in a 12- months period was 11%, of itchy rash on predilectic areas for atopic dermatitis (AD) 8% and 14% for allergic rhinitis (AR). Age group 10 years 0 months- 10 years 11 months: Self reported prevalence of Wh in a 12-months period was 11%, of itchy rash on predilectic areas for AD 14% and of AR 18%. Age group 13 years 0 months- 13 years 11 months: Self reported prevalence of Wh in a 12- months period was 0.6%, of itchy rash on predilectic areas for AD 4.3% and 9.4% of AR.

Conclusion.12-months prevalence of allergic disease symptoms in NPLP are similar as prevalences in other parts of Croatia, and is not lower due to the rural environment as we would expect. On the other hand, the results can not be properly compared, as the last epidemiological study in Croatia was conducted in the year 2009. and none of the studies compared rural and urban environment. Our further research will involve comparison with the data from the city of Zagreb and correlation with risk factors.
ANION-EXCHANGE CHROMATOGRAPHY IN THE BATCH MODE AS A METHOD FOR PURIFICATION OF EQUINE IgGs FROM THE PLASMA

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Background. Immunoglobulin–derived biopharmaceutical products (like antivenoms) should not contain polymer molecules. An appearance of polymers during storage period indicates product instability. It has been shown that purification steps involving precipitation of IgGs during downstream processing of plasma might disturb the tertiary structure of immunoglobulins, making them more prone to polymerization. That is the reason why we investigated the possibilities of purifying IgGs by protocols that render them in solution, avoiding their precipitation and temporary binding to matrices of any kind.

Aim. In a hyperimmune horse plasma (HP), IgGs have higher pl values than most of other plasma proteins, particularly albumin, the most abundant non-IgG protein. Thus, methods of separation on the basis of pl differences have been considered. Driven by the idea to keep IgGs constantly in solution during purification, we investigated the possibility of purifying them by anion-exchange chromatography in the batch mode.

Materials and methods. Toyopearl SuperQ-650M was used as anionic exchanger, and its binding capacity for pure albumin and IgG precisely determined under anticipated experimental conditions for IgG purification from HP. Depletion of albumin from HP was tested by alternating between the following conditions: pH (7 and 8), buffer ionic strength (35 mM and 120 mM buffer), dilution of plasma (5x and 20x) and the quantity of ion exchanger used (volumes of ion exchanger were set at 30% and 60% of the total resin albumin binding capacity). Total protein concentrations were measured using Ehresmann's method and IgG concentrations were determined using ELISA. One-dimensional SDS-PAGE and SEC-HPLC were used to determine the purity of differently treated plasma fractions.

Results. The best purification efficiency was achieved in buffers at pH 8, containing 35 mM NaCl, with IgG purity of 81.2%. However, yields did not exceed 50%. Inadequate separation of IgGs from albumin was connected to the abundance of polymers as determined by SEC-HPLC in the samples where higher NaCl concentration was used. The difference in dilution and ion exchanger quantity had no apparent effect on the separation of IgG and albumin.

Conclusions. This mode of ion-exchange chromatography can be used to purify IgG up to 80% purity under some experimental conditions, but with huge losses (up to 50%). Such results on purity and yield are inferior to other known purification procedures, so further investigation and optimization is to be done.
THE NEW KID ON THE BLOCK - A NOVEL CYTOMEGALOVIRUS’ IMMUNOEVASIN TARGETS NK CELLS IN ORDER TO ESCAPE “MISSING-SELF” RECOGNITION

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Introduction. Cytomegaloviruses (CMVs) are widely spread β-herpesviruses that successfully infect humans and other mammals. CMVs dedicated a large portion of their genome towards immune evasion targeting many aspects of the host immune system, especially towards major histocompatibility complex class I (MHC I molecules) due to its role in CD8+ response. Our comprehensive transcriptomic profiling of murine cytomegalovirus (MCMV) during lytic infection demonstrated that MCMV encodes considerable more transcripts than it was originally thought. Among these, our attention caught a novel, spliced transcript that is expressed to the largest extent throughout the infection called MAT (Most Abundant Transcript). With the total length of 1.7 kb, MAT is located at the right end of a genome, encodes for two proteins, MAT1 and MAT2, and contains a binding site for cellular miRNA-27 on its 3’UTR end.

Aim. The goal of our study was to elucidate the role of MAT transcript during lytic MCMV infection.

Materials and Methods. We used viruses that lack different proportions of MAT transcript to study the role of MAT1 protein in vitro and in vivo.

Results. Here we found that one of MAT encoded proteins, MAT1, has immunoregulatory role and is involved in NK cell recognition of infected cells. The absence of MAT1 leads to the attenuation of the virus in NK cell-dependent manner in mouse strains with different MHC I haplotypes and impairs the interaction between NK cell inhibitory Ly49 receptors and MHC I-m04 complex, necessary for NK cell “missing-self” recognition. Moreover, MAT1 protein regulates maturation of MHC I molecules, quality of MHC I-loaded peptides and surface levels of MHC I thus affecting cytotoxic CD8+ T-cell response. Interestingly, MAT1 is also necessary for specific recognition of MCMV infected cells by activating Ly49 receptors, suggesting co-evolutionary struggle between viral immunoevasin MAT1 and host Ly49 receptors.

Conclusions. A novel MCMV immunoevasin, MAT1, targets inhibitory Ly49 receptors in order to escape NK cells response but has also prompted the evolution of activating Ly49 receptors in response to this viral evasion strategy.
ASSESSING SERUM ALBUMIN CONCENTRATION, LYMPHOCYTE COUNT AND PROGNOSTIC NUTRITIONAL INDEX MIGHT IMPROVE PROGNOSTICATION IN PATIENTS WITH MYELOFIBROSIS

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Background. Primary and secondary myelofibrosis (PMF; SMF) are malignant diseases of the hematopoietic stem-cell characterized by the neoplastic myeloproliferation and strong inflammatory milieu. Prognostic-Nutritional-Index (PNI) integrates information on albumin and absolute-lymphocyte-count (ALC) and reflects inflammatory, nutritional and immune status of a patient. Clinical and prognostic significance of albumin, ALC and PNI in patients with myelofibrosis has not been previously investigated.

Methods. We retrospectively analyzed a cohort of 83 myelofibrosis patients treated in our institution from 2006 to 2017. Albumin, ALC and PNI were assessed in addition to other disease-specific markers.

Results. PMF and SMF patients had significantly lower ALC and PNI and similar albumin compared to controls. Lower albumin was significantly associated with older age and parameters reflecting more aggressive disease biology (anemia, lower platelets, higher LDH, circulatory-blasts, transfusion-dependency, blast-phase-disease), inflammation (higher CRP, constitutional-symptoms) and higher degree of bone-marrow-fibrosis. Lower ALC was significantly associated with lower white-blood-cells (WBC) and lower circulatory-blasts. Low PNI was associated with lower albumin, lower ALC, anemia, lower WBCs, lower serum-iron and lower transferrin-saturation. There was no difference in albumin, ALC and PNI in regard to the driver-mutations. In multivariate analysis adjusted for age and gender, low-albumin (HR=4.61, P=0.001), low-ALC (HR=3.54, P=0.004) and DIPSS (HR=2.45, P=0.001) were able to predict inferior survival independently of each other. Accordingly, low-PNI (HR=4.32, P<0.001) predicted poor survival independently of DIPSS (HR=3.31, P<0.001).

Conclusion. Assessing albumin, ALC and PNI might improve prognostication in patients with myelofibrosis and could assist in recognition of patients under increased risk of death.
SEVERE ATOPIC DERMATITIS WITH HYPOGAMMAGLOBULINEMIA AND CRANIOSYNOSTOSIS IN 6-MONTHS OLD BOY: COMPLEX PRIMARY IMMUNODEFICIENCY OR JUST ATOPY? – CASE REPORT

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Case report. A 6-month-old boy was referred to Neurosurgery department due to metopic synostosis. Neurosurgeon refused to operate the child due to severe atopic dermatitis (AD) and transferred him to Allergology and Immunology Department.

He was the first child of nonconsanguineous parents. His father had asthma during childhood. Past medical history revealed preterm birth due to mother’s cervical insufficiency and AD since the age 2 months. He did not suffer from severe or opportunistic infections in the past. Except skin status, his general condition was not disturbed. He was well-fed, with trigonocephaly, and diffuse AD (SCORAD 70). Other examination findings were normal, no dismorphism. Immunological analysis revealed hypogammaglobulinemia with decreased immunoglobulins (Ig) G1 and G2 subclasses but normal numbers of T, B and NK cells. Patient had normal antibody response to hepatitis B (HB) and normal T-lymphocyte proliferation in the presence of mitogens. He had peripheral eosinophilia, elevated IgE and specific IgE to multiple food allergens. Chest ultrasound revealed thymus. Elimination diet was started with topical treatment od skin and regular intravenous immunoglobulin (IVIG) supstitution every 4 weeks. Differential diagnosis enclosed primary immunodeficiencies (PIDs) with atopic dermatitis (Job's syndrome, Wiskott-Aldrich syndrome) and Netherton's syndrome. Skin biopsy and genetic testing for Job's and Netherthon's syndrome were suggested but not accepted by patient's mother. During follow-up, patient's skin improved and he did not revealed any of severe or opportunistic infection.

Conclusion. We presented the case of the 6 months-old-boy with severe AD and hypogammaglobulismeia who improved on IVIG supstitution, elimination diet and skin care. Good clinical improvement suggest that he had just transient hypogammaglobulinemia with food allergy instead of complex PID. Hypogammaglobulinemia is more frequent in patients with atopic dermatitis compared with the control group, but is is not associated with the severity of atopic dermatitis.
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