7TH INTERNATIONAL CONFERENCE

Cancer Immunotherapy & Immunomonitoring (CITIM)

April 24–27, 2023

VILNIUS, LITHUANIA

Redefining Cancer Therapy

www.CancerITIM.org

Dear Participant,

Welcome to the 7th International **Cancer Immunotherapy and Immunomonitoring Conference**! This meeting is jointly organized by the International and Local Organizing Committees, and we have worked together to make this conference as informative and enjoyable as possible for you. We are looking forward to many superb presentations and discussions over the next few days that will allow you to consider new hypotheses and findings, present your own data and thoughts, and establish new collaborations and friendships. In addition to the outstanding basic and clinical science that will be presented, we hope that magnificent setting of the Crown Plaza Hotel close to the center of beautiful Vilnius will contribute to making this a very delightful and inspiring conference.

During this meeting, we will discuss cellular and molecular mechanisms of immune regulation in the tumor microenvironment that direct initiating and effector phases of antitumor immunity, as well control the efficacy and usefulness of anticancer immunotherapeutic modalities. We will also focus on recent clinical trial results and discuss the lessons we learned from preclinical evaluation of novel therapeutic approaches and immunomonitoring of patients with cancer receiving different modes of therapy. The special emphasis will be placed on topics presenting new ideas on how to improve the efficacy of cancer treatment, including combined chemo-radiation-immunotherapy, immunomodulators, and new targets for therapy. Invited plenary speakers and speakers selected from submitted abstracts, as well as session chairs will provide a framework for discussion. This will offer participants a unique opportunity to evaluate the field of tumor immunology and immunotherapy from different perspectives and might recommend new ways of solving existing problems and difficulties.

We hope that we have assembled one of the preeminent conferences on CITIM topics and we anticipate that the plenary sessions, short talks, poster presentations and after-the-meeting discussions will stimulate a breakthrough in our understanding of how to improve the efficiency of immunotherapy for cancer. If we can be of any assistance, please do not hesitate to call upon us. Please enjoy the meeting!

Sincerely,

CITIM-2023 Organizers

ORGANIZING COMMITTEES

International Organizing Committee Members

- ***Natalia Aptsiauri**, MD, PhD (University of Granada, Spain) (Intl Relationship)*
- ***Andy Hurwitz**, PhD (Repertoire Immune Medicines, USA) (Sponsorship Committee)*
- ***Michael R. Shurin**, MD, PhD (University of Pittsburgh, USA) (Chair)*
- ***Viktor Umansky**, PhD (German Cancer Research Center, Germany) (Vice Chair)*

Local Organizing Committee Members

- ***Vita Pasukoniene**, PhD (NCI, Vilnius Gediminas Technical Univ, Lithuania) (Chair)*
- ***Olha Karaman**, PhD (NCI, Vilnius, Lithuania)*
- ***Neringa Dobrovolskienė**, PhD (NCI, Vilnius, Lithuania)*
- ***Agata Mlynska**, PhD (NCI, Vilnius University, Lithuania)*
- ***Jan Aleksander Krasko**, PhD (NCI, Vilnius Gediminas Technical Univ, Lithuania)*
- ***Emilija Paberalė** (NCI, Vilnius, Lithuania)*
- ***Aurelija Žvirblienė**, PhD (Vilnius University, President of Lithuanian Society for Immunology, Vilnius, Lithuania)*

Scientific and Organizing Advisory Committee

- ***Anahid Jewett**, PhD (UCLA, USA)*
- ***Paul Lehmann**, PhD (CTL, USA)*
- ***Suzanne Ostrand-Rosenberg**, PhD (University of Utah, USA)*

ACKNOWLEDGMENTS

The members of the organizing Committees of **Cancer Immunotherapy and Immunomonitoring** (**CITIM**) Conference express their gratitude and acknowledge the following organizations and companies for their generous support:

- **CTL** (**Cellular Technology Ltd**); CTL Europe GmbH, Bonn, Germany (General Sponsor)
- **Thermo Fisher Scientific**, Vilnius, Lithuania (General Sponsor)
- **CCNF** (**Cure Cancer Now Foundation**), Los Angeles, CA, USA
- **National Cancer Institute**, Vilnius, Lithuania
- **The Lithuanian Society for Immunology**, Vilnius, Lithuania
- **Cureline Baltic, Vilnius**, Lithuania
- **Innovita Clinic and Froceth**, Vilnius, Lithuania
- **Miltenyi Biotec**, Teterow, Germany
- **AstraZeneca**, Vilnius, Lithuania
- **Grida**, Vilnius, Lithuania
- **Immatics Biotechnology GmbH**, Tübingen, Germany
- **Laborama**, Vilnius, Lithuania
- **Linea libera**, Vilnius Lithuania
- **Mabtech**, Nacka Strand, Sweden
- **MSD Oncology**, Vilnius Lithuania
- **NorthSpeed Logistics**, Vilnius, Lithuania
- **SB-PEPTIDE**, Saint Egrève, France
- **Go Vilnius**, Vilnius, Lithuania
- **CREATIVA**, Vilnius, Lithuania
- **Critical Reviews in Immunology** (CRI) journal (Begel House Inc.)
- **Cancer Drug Resistance** (CGR) journal (OAE Publishing Inc.)

CTL (Cellular Technology Ltd.) has been pioneering ELISPOT technology for all aspects of immune monitoring. CTL has been the leader in
"Elevating ELISPOT to an Exact Science"
for over a decade, serving over 2000 clients in academia and industry worldwide and offering unparalleled expertise to the field of immune monitoring.

CTL offers:

- **Contract Research for GLP**, Part 11 compliant, high-throughput ELISPOT testing and other high throughput assays for immune monitoring; we also offer services and logistics for blood sample shipping and cryopreservation using methods which have been proven to retain full functionality of the sample.
- **ImmunoSpot® Analyzers** for fully automated ELISPOT data analysis and streamlined data processing.
- **BioSpot® Analyzers** for high throughput imaging in microplates and automation in data acquisition/analysis.
- **ELISPOT Standardization** including: Standardized protocols and reagents for human PBMC cryopreservation, processing, and testing in high throughput ELISPOT assays; Human PBMC reference sample QC **(Quality Control)** sets for use as benchmarks for assay performance; Positive and negative controls for immune-assays.

  (See our publication for details on our recent successful efforts in a standardized multi-center T cell immune monitoring study utilizing ELISPOT, Zhang et al, J. Immunotoxicology, 2009, 6:227-34.)
- **PBMC Bank for human primary cells,** providing virtually unlimited quantities of ready-to-use, HLA-typed and immune-characterized human PBMCs from over 100 different donors. These cells have been cryopreserved functionally loss-free, and are ideal for high throughput screening in functional assays such as ELISpot, ADCC, ICS, etc.
- **Consultation and Hands-on Training** in all aspects of ELISPOT and high-throughput imaging for immune monitoring assays.

C.T.L.
Cellular Technology Ltd.

Immune Monitoring by ELISPOT

- Cryopreservation of PBMC samples
- Assay development
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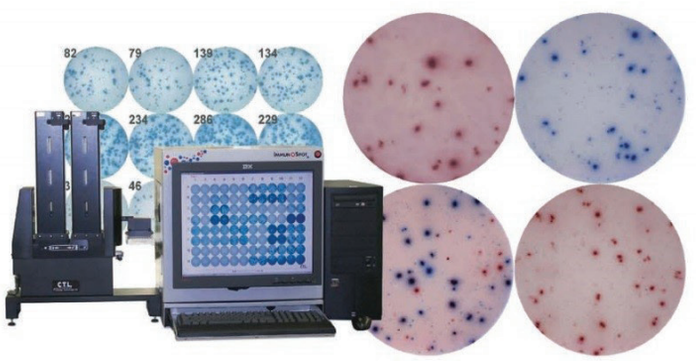
- Assay consultation
- PBMC reference samples
- Serum-free test platforms
- ELISPOT readers



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Fact sheet

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$44B
in revenue

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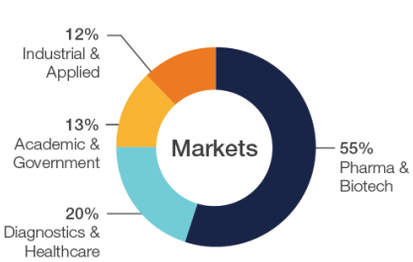

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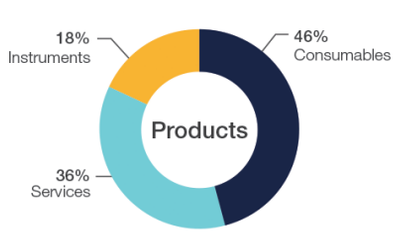
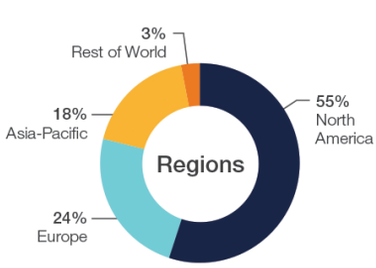


Our global reach

Leading global presence, with ongoing expansion in high-growth and emerging markets



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ORGANIZERS

Organizer

National Cancer Institute is the only specialized oncology institution in Lithuania. The Institute acts as the clinical cancer center, certified and accredited by the Organization of European Cancer Institutes. The patient-oriented international level scientific research works are performed at the Institute with help of biotechnology, nanomedicine, genomics, proteomics and other modern technologies in order to achieve the most important goal - to reduce oncological morbidity, mortality from oncological diseases, and increase the life expectancy of patients, improve their quality of life.
Institute's Laboratory of Immunology is the only group of cancer immunology scientists in Lithuania having the best qualified specialists that have worked for many years in this area. The laboratory carries out research on cancer immunology, cancer immunotherapy, cell technologies. Scientific progress in the area of immunology and immunotherapy provides more and more opportunities to use the knowledge for cancer treatment.

Organizer

The Lithuanian Society for Immunology (LSI) unites specialists in various fields of immunology working in universities, research institutes and clinics. LSI was founded in 1991 and in 1992 LSI became a member of the European Federation of Immunological Societies (EFIS) and the World Association of Immunological Societies (IUIS). Today's LSI members are actively involved in immunological diagnostics, in research of anticancer and antiviral immunology developing and researching therapeutic and diagnostic immunological products, applying immunotherapy to both cancer and allergic diseases, and being very active in COVID-19 pandemic management processes.

PARTNERS

Partner and Gold Sponsor

CTL (Cellular Technology Ltd.) has been pioneering the ELISPOT technology for immune monitoring. For over a decade, CTL's mission has been "Elevating ELISPOT to an Exact Science". Serving over 2,000 clients from academia and industry worldwide, CTL offers unparalleled expertise in immune monitoring.

Media Partner

CDR is a quarterly published journal committed to the rapid publication of high quality, peer-reviewed, original research on pharmacological aspects of drug resistance and its reversal, including drug design, drug delivery, drug distribution and cellular drug resistance.

Partner

The mission of the **Cure Cancer Now Foundation** is to ultimately win the battle against cancer.

Partner

Tour operator **JSC „Senamiescio gidas"** is the pioneer of incoming tourism industry in Lithuania established in 1998. We are the first company in Lithuania that started an open bus "Hop-on, Hop-off" sightseeing tours in Vilnius, Trakai, Kaunas and Klaipėda.
Our company has been awarded twice as the "Most hospitable tour operator in Vilnius" (in 2008 and 2012).
With 24 years of experience in organizing sightseeing and thematic tours across the country and the Baltics, we are experts who understand where you want to go, how to get you there and the priceless experiences you want to have.

CONFERENCE SECRETARIAT

CREATIVA

Conference Secretariat

CREATIVA is a professional service company with longstanding experience in the organization of conferences, meetings, courses.

SUPPORTED BY

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Go Vilnius – the official development agency of the City of Vilnius – provides visitors, investors, relocating talent, entrepreneurs and businesses with everything they need to know about Vilnius.

SPONSORS

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Thermo Fisher Scientific is the world leader in serving science, with annual revenue of over $40 billion. Our Mission is to enable our customers to make the world healthier, cleaner and safer. Whether our customers are accelerating life sciences research, solving complex analytical challenges, increasing productivity in their laboratories, improving patient health through diagnostics or the development and manufacture of life-changing therapies, we are here to support them. Our global team delivers an unrivaled combination of innovative technologies, purchasing convenience and pharmaceutical services through our industry-leading brands, including Thermo Scientific, Applied Biosystems, Invitrogen, Fisher Scientific, Unity Lab Services, Patheon and PPD.
Vilnius site (**Thermo Fisher Scientific Baltics**) develops, manufactures, and distributes products for life sciences, research and diagnostics to the world market. The products are used worldwide to study the structure, expression and diversity of genes and to develop new diagnostic methods for congenital, inherited and infectious diseases.

Cureline BALTIC PRECLINICAL CRO

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CURELINE BALTIC is a global contract research organization (CRO) providing discovery, preclinical and translational platforms and services for the development of therapeutics for oncology, immuno-oncology, and immune-mediated inflammatory diseases.
Our mission is to support the drug discovery pipeline with advanced scientific knowledge, access to innovation, and collaborations with leading biopharmaceutical companies and universities.

INNOVITA KLINIKA | Froceth Frozen cell therapies

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Both **Innovita Clinic and Froceth** specialise in developing, manufacturing and applying Advanced Therapy Medicinal Products (ATMPs), especially in the fields of cancer immunotherapy, regenerative medicine and anti-aging (in cellular level). Combining the knowledge of scientists and doctors of medicine as well as the most advanced technologies and newest achievements in science enables us to obtain an optimal personalized treatment for every patient. Since the products are developed using patients' own tissues and cells, the specific needs of each person are fulfilled without any side effects and allows to achieve the best treatment results.

Miltenyi Biotec

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Miltenyi Biotec is a global provider of products and services that empower biomedical discovery and advance cellular therapy. Our innovative tools support research at every level, from basic research to translational research to clinical application. Used by scientists and clinicians around the world, our technologies enable solutions for cellular research, cell therapy, and cell manufacturing. Our more than 30 years of expertise spans research areas including immunology, stem cell biology, neuroscience, and cancer. Today, Miltenyi Biotec has more than 4,500 employees in 28 countries – all dedicated to helping researchers and clinicians make a greater impact on science and health.

AstraZeneca

Sponsor

AstraZeneca have a bold ambition to provide cures for cancer in every form. We are following the science to understand cancer and all its complexities to discover, develop and deliver life-changing treatments and increase the potential to save the lives of people around the world.
Our Oncology strategy is built with one goal in mind – to push the boundaries of science to change the practice of medicine and transform the lives of patients living with cancer. Our broad pipeline of next-generation medicines, together with our focus on excellence in execution, are aimed at expanding treatment options and improving outcomes for patients with solid tumours and haematological cancers.
We focus on four strategic priorities:
- Pioneering research across six scientific platforms: Tumour drivers and resistance; Immuno-oncology; DNA damage response, Antibody drug conjugates, Epigenetics and Cell therapies;
- Advancing innovative clinical strategies to treat early stages of disease and relapsed or refractory patients;
- Building expertise and leadership in the most prevalent and highest mortality rate tumour types;
- Delivering across our global footprint.

GRIDA

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Grida supports life sciences, medical research and testing laboratories with everything what is needed for their daily routine: from reagents and consumables to general and specialized lab equipment.
Other services: consultations regarding suitable products, equipment calibration and repair.

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immatics

Partner

Immatics is a clinical-stage biopharmaceutical company dedicated to developing advanced immunotherapies that are active against cancer.

LABORAMA
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Laborama specializes in scientific research technologies by deploying innovative flow cytometry, clinical and industrial microbiology, molecular biology and cell biology solutions. The company also provides various supplies for researchers and users of advanced laboratory technologies.
Laborama represent such world-leading manufacturers of laboratory technologies as BD, Qiagen, Lonza, Miltenyi Biotec, Mettler-Toledo Rainin, Corning and other suppliers of cutting-edge technologies for Science and Medicine.
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Mabtech is a Swedish biotech company whose mission is to stimulate life science research, by providing the scientific community with optimized immunoassays and instruments, in particular tools for ELISpot, FluoroSpot, and ELISA. To that end, Mabtech develops and produces a wide range of monoclonal antibodies, kits, peptide pools, and instruments for in vitro applications. Founded in 1986, Mabtech currently operates from its offices in Europe and in North America, in collaboration with a network of distributors around the globe.

MSD Oncology

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We aspire to be the premier research-intensive biopharmaceutical company. We're working to invent a world where cancer isn't just treated but cured.
Our focus on cancer research and treatments.
We're proud to deliver breakthrough innovations in oncology that are helping to extend the lives of patients with certain types of cancer. We're continuing to accelerate what we can achieve for the patients we serve, because everyone needs more ways to treat their cancer and, hopefully, more time.

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SB-PEPTIDE is a French company specialized in peptide synthesis, peptide engineering and peptide analysis. Peptides are 100% made in France, in the company's facilities located in the French Alps, and they are produced and controlled in-house. SB-PEPTIDE can produce all types of peptides with or without modifications, including simple, complex, cyclic, fluorescent peptides, and much more … You decide on the amino acid sequence, quantity, purity, structure's modification, and we synthesize your product. Visit our website to know more about our services !

STRUCTURE OF CITIM 2023 CONFERENCE

CLINICAL AND BASIC ASPECTS OF CANCER IMMUNOTHERAPY AND IMMUNOMONITORING

KEYNOTE LECTURE

PLENARY LECTURES

SPECIAL KEYNOTE PRESENTATIONS

SELECTED SHORT PRESENTATIONS

POSTER PRESENTATION AND DISCUSSION

EXHIBITIONS

BEST POSTER AND TRAVEL AWARDS

REDEFINING CANCER THERAPY by:

- Promoting basic and clinical research in tumor immunology
- Integrating the immunological research and educational activities
- Disseminating information and encouraging international collaborations
- Identifying ways to improve the quality of immunological research and clinical care
- Encouraging and providing training and continuous medical and biomedical education
- Promoting novel preventive, diagnostic and therapeutic modalities and good patient care.

VISITING VILNIUS, LITHUANIA

Dear Colleagues,

It is with great pleasure to welcome you to Vilnius for an exciting scientific meeting CITIM-2023 and to enjoy this vibrant city. Vilnius is an outstanding example of a medieval foundation which exercised a profound influence on architectural and cultural developments in a wide area of Eastern Europe over several centuries.

The legend of Vilnius

Today Vilnius is 700 years old. Legend has it that the city started with a dream Grand Duke Gediminas had on a hunting trip. In his dream, Gediminas saw a huge iron wolf standing on a hill howling as if hundreds of wolves were trapped inside it. When the Grand Duke consulted the court magician Lizdeika, he was told that the wolf was a symbol of a great capital that would one day stand atop that hill. By 1323, Gediminas was already sending letters to European cities inviting merchants and craftspeople to visit the city.

Weather in Vilnius

The rainy season begins in October and gradually turns to snow by December. Winters in Vilnius are usually mild, but you might come to Vilnius when it's truly freezing. Good news: there's still plenty to do despite the cold. The end of January and February are usually the coldest months. You might be lucky and enjoy snow during Christmas, but you can expect even more snow after the New Year. Springtime is lovely with flowers blooming, but you should also expect some rain. It might get hot (25-30°C) in June, but summer kicks into high gear in July. Summer weather is a mix of hot temperatures and sun, or rain showers and colder wind. Don't worry too much; the weather is always perfect to explore Vilnius.

Culture in Vilnius

The magical Old Town of Vilnius holds many secrets and a long history. The cobblestone streets lead you on a romantic walk through the UNESCO World Heritage site. But Vilnius has more tricks up its sleeve. Vilnius is home to many talented contemporary artists, which means you can attend a play or a film by a world-famous Lithuanian director, listen to the best opera singer in the world, or visit the exhibition of an acclaimed contemporary artist. Dozens of free events take place daily in Vilnius, and every month there's a theatre, film, dance, or a culture festival going on. Vilnius is also a hot destination for touring artists, so plan ahead and you might get to see them up close and personal while in town. At night, Vilnius turns into a different city where people dance until sunrise amid a booming club and bar culture.

Enjoy Vilnius during (and after) CITIM-2023 Conference!

14-YEAR ANNIVERSARY

CITIM 2009 – 2023

Kiev – Budapest – Krakow – Ljubljana – Prague – Tbilisi – Vilnius

From 2009:

Natalia Aptsiauri
Arthur Hurwitz
Paul Lehmann
Suzanne Ostrand-Rosenberg
Angel Porgador
Michael Shurin
Galina Shurin
Viktor Umansky
Luca Vannucci
Elena Voronov
Eitan Yefenof

From 2011:

Michal Baniyash
Adit Ben-Baruch
Georgi Guruli
Anahid Jewett
Graham Pawelec
Perthor Straten
Jonathan Weiss
Isaac Witz

CITIM 2023 CONFERENCE SPEAKERS

Ronen Alon
Dept of Immunology
Weizmann Institute of Science, Rehovot, Israel

Natalia Aptsiauri
Dept of Biochemistry, Molecular Biology and Immunology, University of Granada Medical School, Granada, Spain

Michal Baniyash
Hebrew University Hadassah Medical School, Jerusalem, Israel

Adit Ben-Baruch
Dept of Cell Research and Immunology, Ela Kodesz Institute for Research on Cancer Development and Prevention, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel

Rostyslav Bilyy
Danylo Halytsky Lviv National Medical University, Lviv, Ukraine

Sven Brandau
University Hospital Essen, West German Cancer Center, Department of Otorhinolaryngology, Essen, Germany

Ofir Cohen
The Shraga Segal Dept of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben-Gurion University, Israel

Tomer Cooks
The Shraga Segal Dept of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben-Gurion University, Israel

Moshe Elkabets
The Shraga Segal Dept of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Nuray Erin
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Hadassah-Hebrew University Medical Center, Jerusalem, Israel

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Dept of Biomedical Sciences, University of Pennsylvania, Philadelphia, PA, USA

Georgi Guruli
Virginia Commonwealth University Health Center, Div of Urology, Dept of Surgery, Richmond, VA, USA

Christel Herold-Mende
Division of Experimental Neurosurgery, Department of Neurosurgery, University of Heidelberg, Heidelberg, Germany

Arthur A. Hurwitz
Repertoire Immune Medicines, Cambridge, MA, USA

Jadwiga Jablonska
Dept of Otorhinolaryngology
University Hospital Essen, Essen, Germany

Anahid Jewett
Tumor Immunology Lab, Div of Oral Biology and Medicine, The Jane and Jerry Weintraub Center for Reconstructive and Biotechnology, Jonsson Comprehensive Cancer Center,
UCLA School of Dentistry and Medicine, Los Angeles, CA, USA

Olga Karaman
R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology Natl Acad of Sciences of Ukraine, Kyiv, Ukraine
National Cancer Institute, Vilnius, Lithuania

CITIM 2023 CONFERENCE SPEAKERS

Yona Keisari
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Greg Kirchenbaum
Cellular Technology Limited, Shaker Heights, OH, USA

Jan Aleksander Krasko
National Cancer Institute, Vilnius, Lithuania

Andreas Lundqvist
Karolinska Institute, Stockholm, Sweden

Andrii O. Maniak
ME “Rivne Regional Cancer Center” of the Rivne Regional Council, Rivne, Ukraine

Agata Mlynska
National Cancer Institute, Vilnius, Lithuania

Michael Nishimura
Cardinal Bernardin Cancer Center, Loyola University Chicago, Maywood, IL, USA

Rimas J. Orentas
Dept of Pediatrics, University of Washington School of Medicine, Seattle, WA, USA

Suzanne Ostrand-Rosenberg
Div of Microbiology and Immunology, Dept of Pathology, University of Utah School of Medicine, Salk Lake City, UT, USA

Graham Pawelec
Department of Internal Medicine, University of Tübingen Medical School, Tübingen, Germany

Vita Pašukonienė
National Cancer Institute, Vilnius, Lithuania

Angel Porgador
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Licia Rivoltini
Unit of Immunotherapy of Human Tumors, Dept of Experimental Oncology and Molecular Medicine, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

Flavio Andres Salazar Onfray
Disciplinary Program of Immunology, Institute of Biomedical Sciences, Faculty of Medicine, Universidad de Chile, Millenium Institute of Immunology & Immunotherapy, Santiago, Chile

Alexandra Sevko
Prokarium Ltd, London, UK

Michael R. Shurin
Div of Clinical Immunopathology, Dept of Pathology and Immunology, University of Pittsburgh Medical Center and University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA

Yulia V. Shvets
R.E.Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, Natl Acad of Sciences of Ukraine;
NSC “Institute of Biology and Medicine” of Taras Shevchenko National University of Kyiv, Kyiv, Ukraine

Per thor Straten
Center for Cancer Immune Therapy (CCIT), Dept of Hematology, University Hospital Herlev, Copenhagen, Denmark

Marius Strioga
National Cancer Institute
Vilnius, Lithuania

CITIM 2023 CONFERENCE SPEAKERS

Andrei Thomas-Tikhonenko
Dept of Pathology & Laboratory Medicine and Pediatrics, Perelman School of Medicine, University of Pennsylvania, Div of Experimental Pathology and Cancer Pathobiology, Children's Hospital of Philadelphia, Philadelphia, PA, USA

Skaistė Tulytė
Vilnius University Hospital Santaros klinikos, Hematology, Oncology and Transfusion Medicine Center, Vilnius, Lithuania

Viktor Umansky
Skin Cancer Unit, German Cancer Research Center, Heidelberg, Germany

Dario Vignali
Dept of Immunology, Cancer Immunology, University of Pittsburgh and University of Pittsburgh Cancer Institute, Pittsburgh, PA, USA

Sjoerd H. van der Burg
Experimental Cancer Immunology and Therapy, Leiden University Medical Centre, Leiden, Netherlands

Luca Vannucci
Laboratory of Immunotherapy, Institute of Microbiology CAS, v.v.i., Prague, Czech Republic

Elena Voronov
The Shraga Segal Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel

Jonathan Weiss
Cancer and Inflammation Program, Frederick National Laboratory for Cancer Research, NCI, Frederick, MD, USA

Stina Wickström
Karolinska Institutet, Dept of Oncology-Pathology, Stockholm, Sweden

Isaac P. Witz
Department of Immunology, Tel Aviv University, Tel Aviv, Israel

CITIM-2023
PROGRAM

April 24 – 27, 2023

CANCER IMMUNOTHERAPY AND IMMUNOMONITORING
CITIM
April 24–April 27, 2023 Vilnius, Lithuania

1

DAY

April 24, 2023

Monday

Registration **12:00–18:30**

CONFERENCE OPENING CEREMONY14:00–15:00

Conference opening

Michael R. Shurin, Chair, CITIM Organizing Committee

Welcoming & greetings

Vita Pašukonienė, Chair, Local Organizing Committee
Deputy Director of the National Cancer Institute for the Clinic Dr. Marius Kinčius
Vice-Dean for Science and Innovation of Vilnius University Faculty of Medicine Dr. Karolis Ažukaitis
Elected member of Vilnius City Municipality
Aurimas Navys

Special Introductions

Paul V. Lehman (Founder and CEO, CTL)
Michael Nishimura (Loyola University Chicago)
Yona Keisari (Tel Aviv University)

Welcome remarks and information

Viktor Umansky (Vice Chair, CITIM Organizing Committee)

KEYNOTE LECTURE 15:00–16:00

Chair: Natalia Aptsiauri (Granada, Spain)

Angel Porgador, PhD
Professor and Dean, Department of Health Sciences
Albert Katz Chair in Cell Differentiation and Malignant Diseases
The Shraga Segal Department of Microbiology, Immunology and Genetics
Ben-Gurion University of the Negev
Beer-Sheva, Israel

Tumor microenvironment-based activation of synthetic promoters to avoid on-target-off-tumor toxicity in CAR therapy

1 DAY

April 24, 2023

Monday

PLENARY SESSION 1 . . . . . **16:00–18:00**

Immunity and the malignant process

Chair: Suzanne Ostrand-Rosenberg (Salk Lake City, UK, USA)
Chair: Michael R. Shurin (Pittsburgh, PA, USA)

Suzanne Ostrand-Rosenberg **16:00–16:30**
(Salk Lake City, UT, USA)
Tumor-induced immune suppression: The problem is RAGE

Luca Vannucci (Prague, Czech Rep). . . . **16:30–17:00**
Smoldering inflammation, an insidious support to the tumor microenvironment development

Natalia Aptsiauri (Granada, Spain) **17:00–17:30**
Altered tumor antigen presentation and cancer immune escape

COFFEE BREAK **17:30–18:00**

Ron Apte Memorial Lecture **18:00–18:40**

Distinguished Chair: Eitan Yefenof
(Jerusalem, Israel)

Moshe Elkabets, PhD
Professor of Microbiology, Immunology and Genetics
The Shraga Segal Department of Microbiology, Immunology and Genetics
Ben-Gurion University of the Negev
Beer-Sheva, Israel

MEK1/2 inhibition transiently alters the tumor immune microenvironment to enhance immunotherapy efficacy against head and neck cancer

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Welcome reception, Hotel Lobby. **19:00–21:30**

April 25, 2023

2 Tuesday DAY

Keynote Presentation 8:10–8:55

Chair: Viktor Umansky (Heidelberg, Germany)

Sven Brandau (Essen, Germany)
Neutrophils use multiple mechanisms to induce immunosuppression and tumor progression

PLENARY SESSION 2 . . . . . 9:00–12:10
Regulatory pathways in the tumor immuno-environment: Cellular and molecular mechanisms

Chair: Licia Rivoltini (Milan, Italy)
Chair: Marius Strioga (Vilnius, Lithuania)

Zvi G. Fridlender (Jerusalem, Israel) **9:00–9:30**
Interactions and crosstalk between neutrophils and B-cells in the tumor microenvironment

Andrei Thomas-Tikhonenko **9:30–10:00**
(Philadelphia, PA, USA)
Immunoediting of acute leukemia by aberrant splicing

Jonathan Weiss (Frederick, USA) **10:00–10:30**
Metabolic regulation of lipid homeostasis and cancer

Jadwiga Jablonska (Essen, Germany) . . **10:30–10:55**
Neutrophils initiate anti-tumor immune responses in tumor-draining lymph nodes at the early stage of cancer

Andreas Lundqvist **10:55–11:25**
(Stockholm, Sweden)
Regulation of natural killer cell responses in solid tumors

COFFEE BREAK **11:25–11:50**

April 25, 2023

2 Tuesday DAY

PLENARY SESSION 3 . . . . . 11:50–13:10

Regulatory pathways in the tumor neuroenvironment

Chair: Andrei Thomas-Tikhonenko (Philadelphia, PA, USA)
Chair: Zvi G. Fridlender (Jerusalem, Israel)

Isaac P. Witz (Tel Aviv, Israel) **11:50–12:20**
Microglia functions as a double–faced Janus in the progression of melanoma towards brain metastasis

Michael R. Shurin **12:20–12:45**
(Pittsburgh, PA, USA)
Immunoregulatory neuroglial cells in cancer

Nuray Erin (Antalya, Turkey) **12:45–13:10**
Can TRPV1 agonists modulate cancer neuro-environment?

LUNCH BREAK **13:10–14:05**

PLENARY SESSION 4 . . . . . 14:05–16:00

Regulatory pathways in the tumor immuno-environment: Immunosuppressive mechanisms

Chair: Sven Brandau (Essen, Germany)
Chair: Yona Keisari (Tel Aviv, Israel)

Per thor Straten (Herlev, Denmark) **14:05–14:35**
The exercise of TAMing the immune system

Michal Baniyash (Jerusalem, Israel) **14:35–15:05**
The emerging roles of MDSCs in chronic inflammation-associated complications

Christel Herold-Mende **15:05– 15:25**
(Haidelberg, Germany)
Eosinophils are important modulators of an immune-responsive tumor-microenvironment in glioblastoma

Ronen Alon (Rehovot, Israel) . . . . . **15:25 – 15:50**
Escape mechanisms of breast cancer cells disseminated in the lungs

POSTER SESSION. . . . . 16:00 – 17:50

Vilnius overview sightseeing tour . . . . **18:00 – 20:00**
for CITIM-2023 participants

April 26, 2023

3 Wednesday DAY

Keynote Presentation 8:10–8:55

Chair: Arthur Hurwitz (Boston, MA, USA)

Dario Vignali (Pittsburgh, PA, USA)
LAG3: The Third Checkpoint Inhibitor

PLENARY SESSION 5 . . . . . 9:00–10:45

Regulatory pathways in the tumor immunoenvironment: Resistance to immunotherapy

Chair: Moshe Elkabets (Beer-Sheva, Israel)
Chair: Dario Vignali (Pittsburgh, USA)

Ofir Cohen (Beer-Sheva, Israel). **9:00–9:20**
The drug-resistant cell-states in malignant, stromal, and immune cells in metastatic breast cancer – a joint analysis of mutations and transcription, bulk and single-cell RNA-seq

Tomer Cooks (Beer-Sheva, Israel) **9:20–9:40**
Mutant p53 governs tumor microenvironment dynamics via extracellular vesicles

Sjoerd H. Van der Burg **9:40–10:10**
(Leiden, Netherlands)
CD163hi tissue-resident macrophages as drivers of cancer immunotherapy resistance

Adit Ben-Baruch (Tel Aviv, Israel) **10:10–10:40**
Regulation and function of the PD-1/PD-L1 axis in breast cancer

COFFEE BREAK **10:40–11:10**

April 26, 2023

3 Wednesday DAY

PLENARY SESSION 6 . . . . . 11:10–13:00

Cancer immunotherapy: New concepts

Chair: Adit Ben-Baruch (Tel Aviv, Israel)
Chair: Sjoerd H. van der Burg (Leiden, Netherlands)

Michael Nishimura (Maywood, IL, USA). .**11:10–11:40**
Genetically modified T cells for the immunotherapy of cancer

Serge Y. Fuchs (Philadelphia, PA, USA) . .**11:40–12:10**
Omnipresent mechanisms underlying the immune suppression in the tumor microenvironment

Georgi Guruli (Richmond, VA, USA) **12:10–12:40**
Expression of cancer-testis antigens in urological malignancies

Olga Karaman **12:40–13:00**
(Kyiv, Ukraine & Vilnius, Lithuania)
Application of B. subtilis IMV B-7724 lectin in breast and ovarian cancer treatment: preclinical studies

LUNCH BREAK **13:00–14:00**

PLENARY SESSION 7 . . . . . .14:00–16:00

Cancer immunotherapy: New targets and models

Chair: Nuray Erin (Antalya, Turkey)
Chair: Jadwiga Jablonska (Essen, Germany)

Elena Voronov (Beer Sheva, Israel). **14:00–14:25**
Tumor cell-associated IL-1α involves in progression and metastasis of breast carcinoma in mice

Anahid Jewett (Los Angeles, CA, USA). .**14:25–14:55**
Genomic, proteomic and functional attributes of Super charged NK (sNK) cells or genetically engineered Dendritic cells in the treatment of aggressive tumors; role in expansion and increased function of CD8+ T cells

April 26, 2023

3 Wednesday DAY

Jan Aleksander Krasko **14:55–15:20**
(Vilnius, Lithuania)
Umbilical cord blood-derived cytokine-induced killer cells – new options for anticancer immunotherapy

Andrii Maniak (Rivne, Ukraine) **15:20–15:40**
Locally advanced and metastatic cutaneous melanoma: experience of different immunotherapy options.

Stina Wickström (Stockholm, Sweden) . .**15:40–16:05**
Improving ACT therapy through combination with a DC tumor vaccine and protecting NK cells and T cells against oxidative stress

COFFEE BREAK **16:05–16:30**

PLENARY SESSION 8 . . . . . .16:30–18:40

Cancer immunotherapy: Novel approaches

Chair: Michael Nishimura (Maywood, IL, USA)
Chair: Per thor Straten (Herlev, Denmark)

Rimas J. Orentas (Seattle, WA, USA) . . .**16:30–16:55**
Moving from Hematologic Malignancy to Solid Tumors: Pathways to Effective CAR-T Therapy for Pediatric Solid Tumors

Yulia V. Shvets (Kyiv, Ukraine). **16:55–17:15**
Breast cancer and microbiota: in vitro study

Yona Keisari (Tel Aviv, Israel). **17:15–17:45**
Alpha particle mediated radiotherapy (alpha DaRT) of malignant cancer: immune response implications emerging from translational and clinical studies

Alexandra Sevko (London, UK). . . . . .**17:45 – 18:15**
Salmonella typhi strain as a novel immunotherapeutic approach for bladder cancer treatment

Rostyslav Bilyy (Lviv, Ukraine) . . . . . .**18:15 – 18:40**
NEUTROCURE: ROS-on-demand production for modulation of neutrophil functions and antitumor activity

April 27, 2023

Thursday

4 DAY

PLENARY SESSION 9

Best Abstract Presentations 8:30–9:50

Chair: Georgi Guruli (Richmond, VA, USA)
Chair: Vita Pašukonienė (Vilnius, Lithuania)
Benedict Boateng Antuamwine (Essen, Germany)
The biological relevance of tumor-associated neutrophils as critical targets for immunotherapy optimization

Feyza Gul Ozbay Kurt (Mannheim, Germany)
S100A9 and HMGB1 regulates the immunosuppression mediated by MDSC in melanoma

Egle Kvedaraite (Stockholm, Sweden)
NOTCH dependent cooperativity between myeloid lineages promotes Langerhans cell histiocytosis pathology

Laijun Lai (Storrs, CT, USA)
Targeting a novel immune checkpoint molecule TAPBPL in antitumor immunotherapy

Emilija Paberalė (Vilnius, Lithuania)
Modeling of dendritic cell immunotherapy for ovarian cancer

Iryna Tanasiichuk (Kyiv, Ukraine)
Relationship between the senescence and exhaustion status of donor lymphocytes with the cytotoxic activity of CIKs

Kristine Vaivode (Rīga, Latvia)
Transcriptional and functional analysis of CD1c+ human dendritic cells identifies a CD163+ subset priming CD8+CD103+ T cells

Letizia Vitali (Orbassano, Italy)
B7-H3 specific CAR-CIK lymphocytes effectively kill lung cancer cells including chemoresistant cancer stem cells

Best Abstract Award Presentation
Anahid Jewett (Los Angeles, CA, USA)

Special ThermoFisher Sci Presentation 9:50–10:10

Rita Bandariaviciute (Vilnius, Lithuania)
Next generation sequencing for immunology

COFFEE BREAK **10:10–10:40**

April 27, 2023

Thursday

4 DAY

PLENARY SESSION 10 . . . . . .10:40–12:35

Cancer immunotherapy: Novel perspectives

Chair: Anahid Jewett (Los Angeles, CA, USA)
Chair: Jan Aleksander Krasko (Vilnius, Lithuania)

Viktor Umansky (Heidelberg, Germany) .**10:40–11:10**
MDSC generation and their targeting in melanoma

Flavio Salazar (Santiago, Chili) **11:10–11:40**
Immunological essentials for optimizing the clinical effect of tumor cell lysate-based vaccines

Marius Strioga (Vilnius, Lithuania). **11:40–12:05**
Active systemic cancer treatment does not influence antibody immune response to COVID-19 vaccination with mRNA vaccine

Arthur Hurwitz (Cambridge, MA, USA) . .**12:05–12:35**
Decoding the immune synapse for cancer Immunotherapy

LUNCH BREAK **12:35–13:35**

April 27, 2023

4 Thursday DAY

PLENARY SESSION 11 . . . . . .13:35–14:55

Cancer immunotherapy: New approaches in diagnosis and immunomonitoring

Chair: Flavio Salazar (Santiago, Chili)
Chair: Michal Baniyash (Jerusalem, Israel)

Agata Mlynska (Vilnius, Lithuania). **13:35–14:00**
Insights into immune subtyping of ovarian tumors

Graham Pawelec (Tübingen, Germany). .**14:00–14:30**
Peripheral blood biomarkers predicting clinical response to checkpoint blockade in melanoma

Greg Kirchenbaum **14:30–15:00**
(Shaker Heights, OH, USA)
Multiplexed ImmunoSpot® assays enable detailed assessment of *antigen-specific B cell frequency, class usage and functional affinity*

Skaistė Tulytė (Vilnius, Lithuania) **15:00–15:25**
Prognostic and predictive value of circulating T lymphocytes in pancreatic ductal adenocarcinoma

Licia Rivoltini (Milan, Italy). **15:25–15:55**
Myeloid cells and their reprogramming in cancer patient profiling and cure

CONFERENCE CLOSING 15:55–16:10

Concluding Remarks

Natalia Aptsiauri – *Summary*

Banquet **19:00–22:00**

CITIM-2023
ABSTRACTS

CANCER IMMUNOTHERAPY AND IMMUNOMONITORING
CITIM
April 24–April 27, 2023 Vilnius, Lithuania

CITIM-2023 ABSTRACTS

KEYNOTE SPEAKERS

Neutrophils and MDSC as mediators of immune suppression and tumor progression

Sven Brandau
University Hospital Essen, Essen, Germany.

Cancer-related inflammation influences almost all elements of neutrophil biology by tumor-induced or tumor-secreted factors. As a consequence, the production, expansion, recruitment, function and life-cycle of neutrophils is altered in the tumor-bearing host. Many of these changes are also clinically relevant. This relevance is illustrated by strong correlations between high frequencies of intratumoral neutrophils and poor outcome in the majority of human cancers. Recent high-dimensional analysis of murine neutrophils provides evidence for unexpected plasticity of neutrophils in murine models of cancer and other inflammatory non-malignant diseases. New analysis tools enable deeper insight into the process of neutrophil differentiation and maturation. These technological and scientific developments led to the description of an ever-increasing number of distinct transcriptional states and associated phenotypes in murine models of disease and more recently also in humans. At present, functional validation of these different transcriptional states and potential phenotypes in cancer is lacking. Current functional concepts on neutrophils in cancer rely mainly on the myeloid-derived suppressor cell (MDSC) concept and the dichotomous N1-N2 paradigm. In my presentation I will discuss earlier and more contemporary concepts of neutrophils and MDSC in cancer against the background of our own work.

(Reference: Antuamwine BB, Bosnjakovic R, Hofmann-Vega F, Wang X, Theodosiou T, Iliopoulos I, Brandau S. (2022) N1 versus N2 and PMN-MDSC: A critical appraisal of current concepts on tumor-associated neutrophils and new directions for human oncology. Immunol Rev. 2023; 314 (1):250-279, doi:10.1111/imr.13176)

MEK1/2 inhibition transiently alters the tumor immune microenvironment to enhance immunotherapy efficacy against head and neck cancer

Manu Prasad[1,2], Jonathan Zorea[1,2], Sankar Jagadeeshan[1,2], Avital Shnerb[1,2], Sooraj Mathukkada[1,2], Jebrane Bouaoud[3,4], Lucas Michon[3], Ofra Novoplansky[1,2], Mai Badarni[1,2], Limor Cohen[1,2], Ksenia M Yegodyev[1,2], Sapir Tzadok[1,2], Barak Rotblat[5,6], Libor Brezina[1,2], Andreas Mock[7,8], Andy Karabajakian[3,4,9], Jérôme Fayette[3,4,9], Idan Cohen[1,2], Tomer Cooks[1,2], Irit Allon[2,10], Orr Dimitstei[2,11], Benzion Joshua[2,12], Dexin Kong[13], Elena Voronov[1,2], Maurizio Scaltriti[14], Yaron Carmi[15], Cristina Conde Lopez[16], Jochen Hess[17,18], Ina Kurth[16], Luc G.T. Morris[19], Pierre Saintigny[3,4,9], **Moshe Elkabets**[1,2]

[1]The Shraga Segal Department of Microbiology, Immunology, and Genetics, Ben-Gurion University of the Negev; Beer-Sheva, Israel; [2]Faculty of Health Sciences, Ben-Gurion University of the Negev; Beer-Sheva, Israel; [3]Université Claude Bernard Lyon, INSERM 1052, Centre Léon Bérard, Centre de Recherche en Cancérologie de Lyon, Lyon, France; [4]Department of Translational Medicine Oncology, Centre Léon Bérard, Lyon, France; [5]Department of Life Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel; [6]The National Institute for Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer-Sheva, Israel; [7]Department of Medical Oncology, National Center for Tumor Diseases, Heidelberg University Hospital, Heidelberg, Germany; [8]Division of Translational Medical Oncology, National Center for Tumor Diseases, German Cancer Center, Heidelberg, Germany; [9]Department of Medical Oncology, Centre Léon Bérard, Lyon, France; [10]Institute of Pathology, Barzilai University Medical Center, Ashkelon, Israel; [11]Department of Otolaryngology and Head and Neck Surgery, Soroka University Medical Center, Beer-Sheva, Israel; [12]Department of Otorhinolaryngology and Head and Neck Surgery, Barzilai Medical Center, Ashkelon, Israel; [13]School of Pharmaceutical Sciences, Tianjin Medical University, Tianjin, China; [14]Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA; [15]Department of Pathology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel; [16]Division of Radiooncology-Radiobiology, German Cancer Research Center, Heidelberg, Germany; [17]Section Experimental and Translational Head and Neck Oncology, Department of Otolaryngology, Head and Neck Surgery, University Hospital Heidelberg, Heidelberg, Germany; [18]Research Group Molecular Mechanisms of Head and Neck Tumors, German Cancer Research Center, Heidelberg, Germany; [19] Department of Surgery, Memorial Sloan Kettering Cancer Center, New York, NY, USA.

Background: Although the mitogen-activated protein kinases (MAPK) pathway is hyperactive in head and neck cancer (HNC), inhibition of MEK1/2 in HNC patients has not shown clinically meaningful activity. Therefore, we aimed to characterize the effect of MEK1/2 inhibition on the tumor microenvironment (TME) of MAPK-driven HNC, elucidate tumor-host interaction mechanisms facilitating immune escape upon treatment, and apply rationale-based therapy combination immunotherapy and MEK1/2 inhibitor to induce tumor clearance.

Methods: Mouse syngeneic tumors and xenografts experiments were used to analyze tumor growth in vivo. Single-cell CyTOF, flow cytometry, and tissue staining were used to profile the TME in response to trametinib (MEK1/2 inhibitor). Co-culture of myeloid-derived suppressor cells (MDSC) with $CD8^+$ T cells was used to measure immune suppression. Over-expression of colony-stimulating factor-1 (CSF-1) in tumor cells was used to show the effect of tumor-derived CSF-1 on sensitivity to trametinib and anti-programmed death- 1 (αPD-1) in mice. In HNC patients, the ratio between CSF-1 and CD8A was tested to measure associated with clinical benefit to αPD-1 and αPD-L1 treatment.

Results: Using pre-clinical HNC models, we demonstrated that treatment with trametinib delays HNC initiation and progression by reducing tumor cell proliferation and enhancing the anti-tumor immunity of $CD8^+$ T cells. Activation of $CD8^+$ T cells by supplementation with αPD-1 antibody eliminated tumors and induced an immune memory in the cured mice. Mechanistically, an early response to trametinib treatment sensitized tumors to αPD-1-supplementation by attenuating the expression of tumor-derived

CSF-1, which reduced the abundance of two CSF-1R^{+}CD11c^{+} MDSC populations in the TME. In contrast, prolonged treatment with trametinib abolished the anti-tumor activity of αPD-1, because tumor cells undergoing the epithelial to mesenchymal transition (EMT) in response to trametinib restored CSF-1 expression and re-created an immune-suppressive TME.

Conclusion: Our findings provide the rationale for testing the trametinib/αPD-1 combination in HNC and highlight the importance of sensitizing tumors to αPD-1 by using MEK1/2 to interfere with the tumor-host interaction. Moreover, we describe the concept that treatment of cancer with a targeted therapy transiently induces an immune-active microenvironment, and supplementation of immunotherapy during this time further activates the anti-tumor machinery to cause tumor elimination.

Combined approaches to cancer therapy: from synthetic promoters in CAR therapy to artificial reporters for precision cancer medicine

Angel Porgador
The Shraga Segal Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva, Israel.

Harnessing immune effector cells to benefit cancer patients is becoming more and more prevalent in recent years. However, the increasing number of different therapeutic approaches, such as chimeric antigen receptors (CAR) and armored chimeric antigen receptors, requires constant adjustments of the transgene expression levels. In particular, the danger of an overt life-threatening immune response due to the ON-target OFF-tumor CAR cytotoxicity should be considered. We developed synthetic promoters to induce the expression of the CAR immune-effectors within the tumor microenvironment (TME); we will discuss the effect of optimized promoter-responsive elements and minimal promoters on the function of the synthetic promoters in the TME. In addition to CAR-based therapy, immune checkpoint inhibitors (ICI)-based therapy is imperative today in immunotherapy of cancer. Accurate predictive biomarkers of response to ICIs are required for better stratifying cancer patients to ICI treatments. We developed a new concept for a bioassay to predict the response to ICIs that is based on measuring the binding functionality of immunomodulating ligands to their receptor(s) and we will discuss our findings regarding PD1-based ICI therapy.

Immune Inhibitory Mechanisms in in the Tumor Microenvironment

Dario AA Vignali
Department of Immunology, University of Pittsburgh School of Medicine, and Tumor Microenvironment Center, UPMC Hillman Cancer Center, Pittsburgh, PA 15232, USA.

Immunotherapies targeting the PD1/PDL1 pathway have had a major impact on cancer treatment. However, only a proportion of patients respond, and an even smaller proportion exhibit a long-term, durable cure. Several mechanisms of resistance and potential combinatorial approaches will be discussed. Lack of response to inhibitory receptor (IR) blockade therapy and increased disease burden has been associated with circulating, peripheral $CD8^+$ T cell exhaustion, which is defined by poor T cell function linked to increased IR expression (eg: PD1, LAG3, neuropilin-1 [NRP1]). LAG3 is the third IR to be targeted in the clinic, consequently garnering considerable interest and scrutiny. However, persistent antigen exposure in the tumor microenvironment results in sustained PD1/LAG3 expression, contributing to a state of exhaustion manifest in impaired proliferation and cytokine production. Lastly, regulatory T cells (T_{regs}) inhibit beneficial anti-tumor responses. T_{reg} depletion enhances tumor rejection in animal models and the clinic but also leads to substantial adverse events. Thus, approaches have been sought to target Tregs in tumors while limiting systemic autoimmune and inflammatory manifestations.

CITIM-2023 ABSTRACTS

INVITED SPEAKERS

Escape mechanisms of breast cancer cells disseminated in the lungs

Ofer Regev[1], Marina Kizner[1], Francesco Roncato[1], Maya Dadiani[2], Olga Yajuk[3], Stav Kozlovski[1], Nehora Levi[1], Yoseph Addadi[4], Ofra Golani[4], Zvi Granot[3], **Ronen Alon**[1]

[1]Department of Immunology, Weizmann Institute of Science, Rehovot, Israel; [2]Cancer Research Center, Sheba Medical Center, Ramat-Gan, Israel; [3]Department of Developmental Biology and Cancer Research, Institute for Medical Research Israel-Canada, Hebrew University Medical School, Jerusalem, Israel; [4]Life Sciences Core Facilities, Weizmann Institute of Science, Rehovot, Israel.

Malignant and circulating cancer cells derived from primary breast tumors express the leukocyte β2 integrin ligand ICAM-1. High ICAM-1 expression in human breast cancer patients correlates with favorable clinical outcome. We have assessed the in vivo contribution of breast cancer cell expressed ICAM-1 to tumorigenesis and lung metastasis in syngeneic immunocompetent mice hosts using spontaneous and experimental models of the luminal-B BL/6 breast cancer line E0771. The presence of ICAM-1 on E0771 cells did not alter tumor growth or inhibited elimination of primary E0771 tumors by tumor specific CTLs. Both WT and ICAM-1 deficient E0771 cells equally accumulated inside the lung vasculature but the ICAM-1 deficient breast cancer cells developed much larger metastatic lesions than their control counterparts. Strikingly, the vast majority of these cells grew inside vessels where they gave rise to intravascular lung metastasis. ICAM-1 expressing E0771 but not their ICAM-1 deficient counterparts were highly susceptible to elimination by neutrophils but resisted killing by tumor specific CD8 T cells entering the lung vasculature. Furthermore, OVA expressing E0771 cells implanted in the mammary fatpad were readily killed by circulating OT-I CTLs but resisted killing by the same CTLs once reaching the lung vasculature. E0771 escape from neoantigen specific CD8 T cell killing was the result of a rapid lung specific loss of OVA-derived SIINFEKL presentation on tumor cell MHC-1. Collectively, our results indicate that breast cancer cells are selected for expansion in the lungs by downregulating ICAM-1 and eliminating neoantigen peptide presentation on their MHC-I once entering the lung vasculature.

Altered tumor antigen presentation and cancer immune escape

Natalia Aptsiauri

Department of Biochemistry, Molecular Biology III and Immunology, University of Granada Medical School, Granada, Spain.

Cancer immunotherapy based on blocking antibodies targeting PD-1/PDL-1 axis is aimed at activation of anti-tumor T-cell immunity, which requires tumor antigen presentation of T-cells via HLA class I molecules. Loss or downregulation of tumor HLA class I expression represents one of the key mechanisms of cancer immune escape and resistance to immunotherapy. PDL-1/PD-1 blockade enhances the antitumor functions of immune cells, but loss of tumor HLA-I expression could impair the efficacy of the therapy. Previously, in some types of cancer, we observed an association of HLA-I negative/PDL-1 positive tumor pattern with more advanced cancer stage. Blocking PDL-1 in vitro using specific antibodies in some cell lines increases the expression of tumor HLA-I and of T-cell activating ligands. Tumor expression of PDL-1 and other emerging “immune checkpoints” that regulate anti-tumor activity of T-cells and NK cells, are being actively investigated both in patients and in in vitro models. These include LAG3, CD155, CD112, HLA-G, CD70, MICA/B and other immunoregulatory molecules. Here we analyze the expression of a wide panel of “immune checkpoints” in human tumor cell lines of distinct histological origin in correlation with HLA class I expression.

The emerging roles of MDSCs in chronic inflammation-associated complications

Michal Baniyash, Hadas Preiser Ashkenazi, Or Reuven, Kerem Ben Meir, Nira Twaik, Ivan Mikula, Guy Kariv, Mhadi Kurd, Leonor Daniel

The Lautenberg Center for Immunology and Cancer Research, Israel-Canada Medical Research Institute, Faculty of Medicine, The Hebrew University, Jerusalem, Israel.

Site-specific and peripheral chronic inflammation affect immune homeostasis featured by robust changes in the cytokine milieu, composition of immune cells, metabolites, microbiota, and tissue integrity. Myeloid-derived suppressor cells (MDSCs) dominate the environment during chronic inflammation and impair the homeostatic conditions by perpetuating the inflammatory conditions. MDSCs are plastic and serve as environmental sensors that intimately interact with various immune and non-immune cells, pathogens and soluble factors. These ensue in the induction of diverse harmful outcomes associated with immunosuppression, bone loss, tissue damage, predisposition to cancer development and disabling responses to various immune-based treatments. Interfering with MDSC stimulators and/or with their harmful immunosuppressive activities and products and/or protecting their targets (T and NK cells), are aims positioned today at the front line towards the establishment of clinical strategies, especially for cancer treatments. Various aspects of chronic inflammation-related complications will be discussed highlighting the involved key players and the rationale of therapeutic interventions.

Regulation and function of the PD-1/PD-L1 axis in breast cancer

Adit Ben-Baruch[1], Nofar Erlichman[1], Tamir Baram1, Tsipi Meshel[1], Dina Morein[1], Benny Da'adoosh[2]

[1]The Shmunis School of Biomedicine and Cancer Research, George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv, Israel; [2]Blavatnik Center for Drug Discovery, Tel Aviv University, Tel Aviv, Israel.

Therapies targeting the PD-L1/PD-1 axis have been recently introduced to triple-negative breast cancer (TNBC) with limited efficacy, suggesting that this axis promotes tumor progression through mechanisms other than immune suppression. Here, we over-expressed WT-PD-L1 in human TNBC cells (express endogenous PD-L1) and in luminal-A breast cancer cells (no endogenous PD-L1 expression) and demonstrated that cell-autonomous PD-L1 activities lead to increased tumor cell growth, invasion and release of pro-metastatic factors (CXCL8, sICAM-1, GM-CSF). These activities were promoted by PD-1, and were inhibited by mutating S283 in PD-L1. Studies with T cell-deficient mice demonstrated that cell-autonomous WT-PD-L1 activities in TNBC cells increased tumor growth and metastasis compared to knock-out (KO)-PD-L1 cells, whereas S283A-PD-L1-expressing cells had minimal ability to form tumors and did not metastasize. From the mechanistic perspective, we found that invasion of WT-PD-L1-cells required signaling by chemokine receptors CXCR1/2, CCR2 and CCR5 through autocrine circuits involving CXCL8, CCL2 and CCL5. Moreover, our findings revealed key roles for STAT1 and STAT3 in regulating the autonomous, but not the PD-1-induced activities of PD-L1 in breast cancer cells; this was demonstrated by increased phosphorylation of STAT1 and STAT3 in WT-PD-L1-expressing cells compared to S283A-PD-L1 cells, and by the ability of siSTAT1/siSTAT3 to down-regulate the invasion of PD-L1-expressing cells (compared to control cells), and their ability to produce CXCL8. Overall, our findings reveal autonomous and PD-1-induced tumor-promoting activities of PD-L1 that depend on S283, on chemokine circuits and/or STAT1/STAT3 activation. These results suggest that TNBC patients whose tumors express PD-L1 could benefit from therapies that prevent immune suppression by targeting PD-1/CTLA-4, alongside with antibodies to PD-L1, which would allow maximal impact by mainly targeting the cancer cells.

NEUTROCURE: ROS-on-demand production for modulation of neutrophil functions and antitumor activity

Rostyslav Bilyy on behalf on NeutroCure Consortium

Danylo Halytsky Lviv National Medical University, Lviv, Ukraine.

Reactive oxygen species (ROS) have key functions in healthy organism such as redox signaling for regulation of cell growth, triggering formation of neutrophil extracellular traps (NETs), and modulation of inflammation. Since in high concentration ROS are damaging to tissues, nature has evolved precise mechanisms to control their generation at the required time, concentration and space, proximal to their target. Disturbance of these mechanisms leads to aberrant ROS production that causes uncontrolled inflammation, occurs in myeloablation caused by radio- or chemotherapy and is a crucial feature of cancer cell phenotype as well as autoimmunity. Despite the damaging properties of ROS it is a paradox that pharmaceutical ROS amplifiers can reverse ("cure") many pathologic features. For example, ROS-induced cancer cell killing inhibits cancer growth, ROS-induced deactivation of T-cells and NETs generation contributes to resolution of inflammation, and ROS-induced boosting of haematopoiesis can relieve myeloablation.

NeutroCure Consortium is developing novel ROS-enhancer for targeting action against tumors both directly (since tumor cells has high amount of ROS and can not compensate their overproduction) and indirectly by targeting neutrophils, including tmor-assosiated neutrophils, reportedly produced high levels of internal ROS. Using an innovative approach based on the multiple-trigger prodrug activation, this consortium is aimed to develop safe ROS amplifiers capable of boosting ROS specifically in abnormal polymorphonuclear neutrophils associated with cancer, uncontrolled inflammation and relevant for myeloablation without affecting normal cells. NeutroCure consists of 6 European academic partners and an SME who will promote commercialization of the new drugs.

Here we demonstrate data of few created prodrug molecules being able to selectively accumulate in mitochondria, lysosomes, ER of cancer cells and enhancing ROS-production level to those causing cell death. In vivo implication in NK/Ly cells frown in lymphoma or sarcoma tumors in mice were shown to be effectively eliminated by the action of coomarine- and amminoferocene-based ROS-enhancers. The level of myeloid-derived suppressor cells (MDSC) was also altered in mice following the therapy with ROS-enhancers.

Summarizing, we would like to attract the attention of scientific community to the possibility of novel ROS-enhancing drugs targeting tumor cells and aberrant neutrophils.

The drug-resistant cell-states in malignant, stromal, and immune cells in metastatic breast cancer – evolutionary convergence of genomic and transcriptomic adaptations

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The commonplace efforts to study the molecular underpinnings of drug resistance in cancer focus on mutations. While genomics remains indispensable, they cannot capture epigenetic mechanisms, fail to reveal the cellular strategies that underscore the phenotype, and are underpowered when facing the intractable challenge of many low-frequency mutations. We address this challenge by uncovering evolutionary convergent drug-resistant transcriptional states in Metastatic Breast Cancer (MBC). (1) Using whole-exome sequencing pre and post-progression, we determine evolutionary acquired mutations in clinical-grade MBC and highlight key resistance modalities, including ER and Growth-Factor Receptors (GFR) pathways activated states. (2) Using RNA-seq in perturbed cells and in tumors, we infer mutation-associated transcriptional states and uncover the main characteristics of the GFR-activated state, including ER-reprogramming, predominant MAPK signaling, and stem-like mesenchymal features. (3) Using in-vitro multi-drug functional characterization, we reveal differential responses to clinically relevant drugs and substantiate the functional implications of the convergent drug-resistant states. (4) Studying transcriptional programs in clinical grade samples, we uncover drug-specific differential responses. (5) Leveraging >100k single-cell RNA-seq (scRNA-seq), we underscore the central transcriptional programs in malignant MBC, recapitulating our bulk-cohort results at the single-cell level. (6) Finally, comparing the tumor microenvironment among metastatic sites with scRNA-seq, we reveal a site-specific microenvironment with substantial immune modulation in metastatic liver sites. Our study represents a generalizable approach to infer malignant states in clinical samples and predict drug-response as a step towards precision oncology at the cell-state level.

Mutant p53 governs tumor microenvironment dynamics via extracellular vesicles

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Mutations in TP53 are considered one of the most frequent genetic alterations in human cancer. Besides the abrogation of the wild-type (WT) p53-mediated tumor suppression, a distinct set of missense mutations was reported to endow mutant p53 proteins with novel activities termed gain-of-function (GOF). Even though mutations in TP53 are typically thought to arise in the tumor cells rather than in the stroma, the non-cell-autonomous effects of these mutants over the tumor microenvironment are poorly understood.

Extracellular vesicles (EVs) shed by cancer cells play a major role in mediating the transfer of molecular information by reprogramming the tumor microenvironment (TME). Here, we demonstrate that GOF mutant p53 proteins can be transferred via EVs to neighboring cancer cells and to macrophages, thus modulating them to release tumor supportive cytokines. Our data from pancreatic, lung, and colon carcinoma cell lines demonstrate that the mutant p53 protein can be selectively sorted into EVs. More specifically, mutant p53 proteins in EVs can be taken up by neighboring cells and mutant p53 expression is found in non-tumor cells in both human cancers and in non-human tissues in human xenografts. Our findings shed light on the intricate methods in which specific GOF p53 mutants can promote oncogenic mechanisms by reprogramming and then recruiting non-cancerous elements for tumor progression. Separately, in two lung cancer cohorts, we identified a signature of microbiome members associated with p53 mutations. Acidovorax Temperans, a Gram negative bacterium, was found to be abundant in tumors of patients with mutant p53. There is a significant increase in tumorigenesis in animals inoculated with Acidovorax temperans as compared to Sham treated animals. These preliminary data indicate that Acidovorax temperans contributes to lung tumorigenesis in the presence of activated K-Ras and mutant p53. OMVs shed by Acidovorax temperans promoted inflammatory signaling in lung carcinoma cells and elevated CD47 expression on tumor cells and SIRPα levels on macrophages.

Conclusions: We show a microenvironmental role for specific “hot-spot” GOF p53 mutants tightening the interaction between the tumor cell and the immune compartment. Mutant p53 facilitates cellular interactions within the tumor microenvironments mediated by vesicles.

Can TRPV1 agonists modulate cancer neuro-environment?

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Capsaicin-sensitive sensory neurons are sensitive to capsaicin that stimulates the transient receptor potential vanilloid 1 (TRPV1) channels that are highly expressed by unmyelinated fibers. Capsaicin-sensitive sensory nerves not only have sensory (afferent) functions but also have local effector functions through releasing neuroactive peptides. Inactivation of sensory neurons expressing TRPV1 enhances breast cancer metastasis. Sensory neurons have profound effects on immune response to a wide range of diseases including cancer. Hence, activation of sensory nerves using feasible approaches such as specific TRPV1 agonists may inhibit breast cancer metastasis through neuro-immune pathways. TRPV1 agonists are considered for the treatment of pain and inflammatory diseases. We recently examined the effects of olvanil, a TRPV1 agonist, on metastasis of breast carcinoma. Olvanil is an archetype of a non-pungent vanilloid agonist that activates TRPV1 more slowly than capsaicin and has a comparable potency. These features of olvanil are likely to provide better tolerability off the compound compared to capsaicin. Olavanil markedly suppressed metastasis that that was mainly due to activation of neuro-immune pathways since olvanil dose used here is not high enough to directly activate immune cells. Local and systemic immune responses are critical to inhibit cancer growth and metastasis. As immune editing states, initial ability of immune cells to eliminate cancer cells decreases over time allowing growth of highly aggressive tumors. For most tumors, TRPV1 mediated neuro-immune regulation of tumor microenvironment enhances anti-tumoral immunity by inhibiting chronic inflammation. Immune cells express TRPV1, though at a lower level, and consequences of TRPV1 activation of immune cells might be different from activation of neuronal TRPV1 and resulting neuro-immune responses during tumor growth and metastasis. Recently we observed that mix leucocyte cultures of tumor-bearing mice secrete markedly higher levels IL-6 and TNF-α, and lower levels of IFN-γ in 4T1-related breast carcinoma model. Interestingly TRPV1 agonists differentially alter cytokine secretion from mix leucocyte culture in tumor-bearing mice compared to control mice when directly added to in-vitro to leucocyte culture. Specifically, TRPV1 agonists markedly increased IFN-γ response in control mice while the opposite occurred in tumor-bearing mice. TRPV1 agonists markedly enhanced IL-17 secretion in control mice while they did not alter the levels of IL-17 secretion in tumor bearing mice. Lastly, TRPV1 agonists increased IL-6 secretion in tumor-bearing mice but not in control mice. Hence activation of TRPV1 channels in the absence of aggressive inflammatory carcinoma may enhance immune response against newly formed precancerous lesions by increasing IFN-γ and acute inflammatory response e.g., by increasing IL-17 secretion. On the other hand, the opposite may occur in the presence of highly aggressive and inflammatory carcinoma such that activation TRPV1 channels of immune cells may create excessive inflammatory conditions. Hence under certain pathological conditions e.g., in the presence of inflammatory metastatic carcinoma, doses of TRPV1 high enough to activate TRPV1 in immune cells may have detrimental consequences and may also partly explain tumor-prompting effects of TRPV1 agonists under certain conditions.

Interactions and crosstalk between neutrophils and B-cells in the tumor microenvironment

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The immune system undergoes in the context of cancer a dramatic modulation which is regarded as an “immunosuppressive switch” during which the tumor-infiltrating immune cells are polarized and modulated to support tumor progression. Tumor-associated neutrophils (TANs) have been identified as key players in multiple aspects of cancer, from malignant transformation to tumor progression, modification of the extracellular matrix (ECM), cell migration, immunosuppression and angiogenesis. A major mechanism through which neutrophils have been suggested by us and others to affect tumor growth involves the interaction and subsequent modulation of other infiltrating immune cells.

B-cells have been found to infiltrate various cancer types and play a role in tumor immunity, offering new immunotherapy opportunities. However, thus far, conflicting evidence has accumulated about their role in the cancerous process, since B-cells have been shown to facilitate tumor progression on the one hand, but also to retain the ability to inhibit tumour growth on the other hand. A possible explanation for this duality could be the presence of different subsets of B-cells, such as regulatory B-cells (B-regs), T2-MZ precursor B-cells and antibodies-secreting plasma cells, presenting different functions and abilities. The specific impact of tumor-associated neutrophils (TANs) on B-cells has largely been overlooked.

In our study, we investigated the role of TANs in the modulation and recruitment of B cells into the tumor. We found prominent differences between TANs and bone marrow neutrophils in their capability to induce B cell chemotaxis and identified the mechanisms through which TANs promote the recruitment of B cells to the tumor microenvironment. Unexpectedly we identify TNFα as the major cytokine mediating B-cell chemotaxis by TANs. Furthermore, we found than TANs drive the differentiation of B cells into plasma cells, following their recruitment into the tumor, resulting in IgG production. This process is T-cell-independent, and at-least partially mediated by membranal BAFF on TANs. Our study therefore demonstrates for the first time that TANs drive the recruitment and modulation of B-cells into plasma cells in the tumor microenvironment, hence opening new avenues in the targeting of the immune system in cancer.

Omnipresent mechanisms underlying the immune suppression in the tumor microenvironment

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The evasive strategies utilized in the tumor microenvironment (TME) of solid tumors are central to their ability to escape from immune surveillance and to resist immune therapies. The sheer redundancy of cellular (Treg, MDSC, TAM, etc.) and acellular (tryptophan metabolites, suppressive cytokines, adenosine, etc.) factors hinders our ability to target them. This consideration prompts therapeutic exploration of prevalent and ubiquitous mechanisms enabling immune suppression and establishment of the immune-privileged niches within the TME. Identification of such mechanisms remains of major theoretical and translational importance. Here we describe how inactivation of the type I interferon (IFN1)-dependent expression and function of cholesterol 25-hydroxylase (CH25H) occurs in malignant and diverse benign intratumoral cells. Loss of IFN1 signaling supports immunosuppressive activities of MDSC and Treg cells while undermining viability and activities of the cytotoxic T lymphocytes (CTLs). Whereas CH25H-deficient malignant cells evade recognition by the anti-tumor CTLs by autophagy-driven downregulation of the MHC-I molecules, loss of CH25H in the intratumoral dendritic cells prompts excessive lysosomal degradation and deficient cross-presentation of tumor antigens. Finally, downregulation of CH25H in CTLs predisposes them to trogocytosis, fratricidal killing and exhaustion. Therapeutic disruption of these events reactivates native and CAR-bearing CTLs and improves the efficacy of immune therapies.

Expression of cancer-testis antigens in urological malignancies

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Cancer testis antigens (CTA) are a group of highly immunogenic (in many cases) tumor-associated antigens expressed in embryonic stem cells and testicular germ cells, which have attracted interest as potential targets of immunotherapy. They are aberrantly expressed in various tumors, including urologic malignancies. Decreased expression of these immunogenic antigens might confer low immunogenicity, allowing tumors to escape immune monitoring and grow unimpeded.

In this study, we evaluated the expression of various CTAs in renal cell carcinoma, bladder cancer, and prostate cancer tissues. We obtained data from the different cohorts of patients from The Cancer Genome Atlas (TCGA). The expression of 13 different CTAs (ACRBP, AKAP4, CCNA1, CEP55, CTCFL, MAGE-A4, ODF4, PAGE4, SPA17, SSX2, TEX14, TEX15, TSGA10) was evaluated. There were 538 patients with clear cell renal cell carcinoma (with 72 corresponding normal renal tissue samples), 411 patients with urothelial carcinoma of the bladder (19 corresponding normal urothelial tissue samples) and 502 patients with the adenocarcinoma of the prostate (52 samples of the normal prostate for comparison). We also obtained surgical specimens from radical nephrectomies and cystectomies from the Virginia Commonwealth University's tissue bank.

Evaluation of the data from TCGA demonstrated different expressions of the CTAs in different tumors. In kidney cancer samples, the levels of seven out of 13 CTAs were decreased, while 4 were unchanged and only 2 were increased in comparison to normal renal tissue. In bladder cancer samples, the expression of only one CTA (TEX15) was decreased, while 6 were increased and 6 were unchanged. Concerning prostate adenocarcinoma samples, the level of 4 CTAs was decreased, 4 unchanged and 5 increased. CEP55 was one CTA which demonstrated significantly increased expression in all urological malignancies examined.

For postoperative samples, quantitative polymerase chain reaction was performed to compare CTA expression in normal and tumor tissues. Expression of CTAs was significantly increased in high grade kidney tumors compared to normal tissues ($p<0.001$) and low grade tumors ($P<0.001$). Comparing expression in Fuhrman grade IV tumors to expression of tumors with Fuhrman grades I-III, 12 of the 13 CTAs studied showed significantly increased expression. In contrast, expression of CTAs was significantly decreased in tumor samples compared to normal tissue ($p<0.001$). In the bladder cancer specimens analyzed, expression of the CTAs studied was significantly less than in the tumor-adjacent control samples.

In conclusion, our results showed mostly decreased expression of examined CTA in human cancer tissues, which may help cancer cells to avoid detection by immunocompetent cells and escape immune surveillance. Use of hypomethylating agents, which have been shown to increase the expression of CTAs in various tumors, may have therapeutic value in these patients. Robust expression of these highly immunogenic genes in cancer tissue may be used to design targeted vaccine therapies with possible tailoring to the individual patient depending on expression of a particular gene.

Eosinophils are important modulators of an immune-responsive tumor-microenvironment in glioblastoma

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Eosinophils, bone marrow-derived granulocytes involved in allergic asthma and autoimmunity, have attracted increasing attention due to their assumed role in effective responses to immune checkpoint blockade in some extracranial tumors. However, the role of this rare type of immune cells in immunologically cold tumors such as glioblastoma (GBM) has not been studied so far. When quantifying immune infiltrates and levels of 30 immune-relevant cytokines in 60 matched pairs of human primary and recurrent GBM, strongest correlations (r= 0.71-0.93) were observed for the eosinophil-associated cytokines Eotaxin and IL-5 as well as for TNF-α, IFN-γ and IL-2, which are important cytokines for cytotoxic T cell responses. A combined score of these cytokines (Eo score) turned out to be an independent prognostic marker for improved overall survival of GBM patients. Furthermore, multicolor immunofluorescent staining revealed that Eo score high GBM are significantly higher infiltrated by effector T cells and show a significantly lower infiltration with anti-inflammatory M2-like tumor-associated macrophages / microglia cells (M2-TAM) in recurrent GBM. Flow cytometry analysis of GBM-derived single cell suspensions confirmed a positive correlation of higher eosinophil numbers with higher numbers of tumor-infiltrating T cells, while an inverse relation between eosinophils and monocytes could be demonstrated in the TCGA data set. Spatial transcriptomic data of human GBM confirmed that areas with a higher eosinophil infiltration showed higher numbers of T cells and pro-inflammatory M1-TAM, lower numbers of M2-TAM and a remarkable vessel normalization. Finally, in a newly developed patient derived GBM organoid model we could corroborate the functional impact of eosinophils on the tumor microenvironment. An increase of eosinophil numbers in these patient avatars resulted in a significantly decreased number of M2-TAM. Altogether, tumor-infiltrating eosinophils seem to be a novel type of immune-modulator in GBM and may enhance immune responses by shaping the tumor niche from an immunosuppressive to an immunopermissive microenvironment.

Decoding the Immune Synapse for Immunotherapy

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Immunotherapy is heavily dependent on understanding T cell recognition of target cells. For autoimmunity, this involves identifying self-antigens that contribute to disease pathogenesis and developing strategies that either prevent or reverse the autoimmune process. For cancer, an over-arching goal is to generate potent T cell responses directed against tumor-associated antigens (TAAs), with preference for TAAs that are more tumor-specific and strongly immunogenic. Repertoire has developed platform-enabling technologies that allow elucidation (or „decoding“) of the immune synapse, which support drug development for both autoimmunity, cancer, and other immune-mediated diseases. DECODETM Synapse (CIPHER) uses barcoded peptide/MHC tetramers and single cell sequencing to probe the broad repertoire of antigens recognized by disease-relevant T cells. DECODE TCR (MCR) is a mammalian expression-based platform for de-orphaning TCRs based on screening antigens known or predicted to be expressed in target tissues. DECODE Antigen (MEDi) supports these technologies by assessing MHC binding of putative protein-derived peptides. We have previously reported on the use of these technologies to identify disease-relevant antigens in infectious disease and are now applying them to antigen discovery for both oncology and autoimmunity settings. Integration of these antigens and TcRs into Repertoire‘s therapeutic modalities (e.g., lipid nanoparticles that promote tolerance and TcR-instructed therapies) will support our drug development strategies.

Neutrophils as regulators of adaptive anti-tumor immune responses in cancer

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Tumor-associated neutrophils contribute to cancer progression via multiple mechanisms, such as support of angiogenesis or the regulation of anti-tumor immune responses. All these processes were described for the primary tumor site; however, the initiation of effective anti-tumor immune responses takes place rather in tumor-draining lymph nodes (TDLNs) than in tumor tissue itself. Therefore, we have studied the role of neutrophils in the regulation of adaptive immune responses in such lymph nodes. We observed that neutrophils form a substantial population of myeloid cells in TDLNs during progression and are the first one to arrive there after tumor setting. Interestingly, at the early metastatic-free (N0) stage of cancer, tumor-associated neutrophils transmigrate to lymph nodes, develop antigen (AG)-presenting phenotype and stimulate T-cells. At later stages of tumor progression, the presence of LN metastases (N1-3) triggers the development of PD-L1+ immunosuppressive neutrophils, which repress T-cell responses in contact-dependent manner. Therefore, the accumulation of neutrophils in T-rich zones of TDLNs in N0 constitutes a positive predictor for 5-years survival of cancer patients, while the increased numbers of neutrophils in TDLNs in N1-3 stages serve poor prognosis predictor. These results suggest a dual-edged role of neutrophils as essential regulators of anti-cancer immune responses and argue for approaches fostering anti-cancer activity of these cells during cancer immunotherapy.

Genomic, proteomic and functional attributes of Super charged NK (sNK) cells or genetically engineered Dendritic cells in the treatment of aggressive tumors; role in expansion and increased function of CD8+ T cells

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We have previously demonstrated that natural killer (NK) cells are the main immune effectors mediating selection and differentiation of a number of stem-like/ poorly differentiated/dedifferentiated (SL/PD/DD) tumors via killing and secreted or membrane-bound interferon-γ and tumor necrosis factor–α, respectively. In this presentation, we will present the recent advances on genetically engineered CD34+ derived Dendritic cells (DCs) in increased activation of cytotoxic immune effectors, and targeting and elimination of aggressive tumors. Treatment of SL/PD/DD pancreatic, oral, glioblastoma, breast, melanoma and ovarian tumors with sNK cells or CD34+ derived DCs results in growth inhibition and curtailment of tumor metastasis in in vitro and in vivo experiments. Moreover, we will present an overview of our findings on sNK and engineered CD34+ derived DC cells in terms of their biology and their significance in selection, differentiation and targeting of SL/PD/DD tumors using various in vitro and in vivo studies conducted in nonobese diabetic/severe combined immunodeficiency (scid)/interleukin-Rγ– –, humanized–bone-marrow/liver/thymus (hu-BLT) mice, and those with human cancer patients. Studies on single cell RNAseq analysis, western blot, proteomics and functional analysis demonstrated sNK cells as unique populations of NK cells with significant increase in activating receptors, decreased inhibitory receptors, increased in memory related phenotype, upregulations of anti-apoptotic and co-stimulatory genes and elevation in all known death inducing effectors among many other attributes. Engineered CD34+ derived DCs due to the genetic increase in CD93, CXCL13, CD40L or 4IBBL have all the attributes of functional primary DCs with significantly elevated capacity to bind, recruit, co-stimulate, and present antigen to T cells. Furthermore, we will present recent advances in sNK cell expansion and therapeutic delivery, and discuss the superiority of allogeneic sNK cells for cancer treatment in regards to manufacturing, regulatory and ongoing clinical trials. We will discuss the potential loss of NK cell numbers and function at neoplastic and preneoplastic stages of tumorigenesis in patients and BLT-mice as potential mechanisms for pancreatic cancer induction and progression. The identification, characterization and use of disease specific personalized probiotic bacteria formulated to activate NK cells and DCs in a number of diseases, including reversing bone loss induced by the pancreatic and oral cancers and restoring bone density and structure will also be discussed. We will demonstrate synergistic targeting of SL/PD/DD tumors in hu-BLT mice by sequential treatment with sNK cells and chemotherapy or sNK with check point inhibitors, or sNK with CD34+ derived DCs. Therefore, sNK cells and engineered DCs should be placed highly in the armamentarium of tumor immunotherapy due to their indispensable role in targeting SL/PD/DD tumors, and more importantly in expansion and functional activation of CD8+ T cells, and targeting gene mutations and knock downs in mice and humans.

Application of *B. subtilis IMV B-7724* lectin in breast and ovarian cancer treatment: preclinical studies

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Lectins are powerful modulators of cell signaling related to the glycosylation of most proteins and lipids in the human body. Increased activity of transferases, in particular fucosyltransferase and sialyltransferase, is shown in tumor cells, due to which the surface of malignant cells has a clear glycoconjugate signature. This feature can be used for targeting such cells with specific lectins. To date, it has been established that the antitumor effects of lectins (regardless of their origin) are realized either through a direct effect on tumor cells through such mechanisms as apoptosis, autophagy, inhibition of proliferation, or indirectly through the activation or modulation of the immune cell and tumor interplay. Recently, lectin-producing bacteria are used to obtain lectins, among which representatives of the genus Bacillus deserve special attention. These bacteria are able to secrete lectins into the growth medium, which greatly simplifies the harvesting process.

The aim of this work was to investigate the antitumor effects of the lectin obtained from the culture liquid of the lectin-producing B. subtilis strain IMV B-7724 in *in vivo* and *in vitro* models. *In vitro* models included breast cells with different molecular subtypes and sensitivity to doxorubicin: MCF-7 (p53wt, ER+, HER-2/neu-), MCF-7/Dox, and MDA-MB-231 (p53mut, ER-, HER-2/neu -). *In vivo* models focused on sensitive and resistant to cisplatin strains of Geren's carcinoma (rat model) and Ehrlich's carcinoma (murine model). A lectin characterized by high affinity to N-acetylneuraminic (1.9 mM) and N-glycolylneuraminic (7.5 mM) sialic acids, D-glucuronic acid, and fructose-1,6-diphosphate was used.

Lectin immunotherapy in mice and rats with model tumors demonstrated an antitumor effect and contributed to the preservation of the antitumor activity of macrophages and natural killer cells in the late stages of tumor growth. It is important that in the case of a cisplatin-resistant tumor, the lectin demonstrated a pronounced antitumor effect in monotherapy, and in a cisplatin-sensitive tumor, such immunotherapy is better combined with cisplatin itself. In experiments *in vitro*, the lectin induced caspase-dependent apoptosis, especially in MCF-7 cell lines, independent of sensitivity to doxorubicin.

Alpha particle mediated radiotherapy (alpha DaRT) of malignant cancer: immune response implications emerging from translational and clinical studies

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Destruction of tumors by photon and particle radiation can release tumor antigens and danger signals and thus turn the tumor into its own vaccine. Alpha particles are a lethal form of radiation whose short range limits its use for cancer treatment. We developed an intra-tumoral alpha radiation-based tumor ablation treatment termed *Diffusing Alpha emitters Radiation Therapy* (DaRT). Radium-224 loaded stainless-steel wires (DaRT seeds) inserted into the tumors, release by recoil short-lived alpha-emitting atoms, which disperse in the tumor and spray it with highly destructive alpha particles.

Results:

1. Treatment of solid tumors in mice with DaRT seeds caused significant retardation of tumor growth, cure, and reduced lung metastases in animals bearing squamous cell, lung, pancreatic, colon, prostate or breast mouse or human derived tumors. The effect was reliant on the extent of radioactive atoms spread inside the tumor and the sensitivity of the tumor cells to alpha particles.
2. Higher levels of tumor control were evident when treatment with Ra-224 seeds was combined with chemotherapy, inhibitors of DNA damage repair, or antiangiogenic agents.
3. Treatment of tumors in mice triggered a robust and specific anti-tumor immunity.

3.1 DaRT in combination with the immune stimulator, CpG, cured mice with highly metastatic tumors DA3 adenocarcinoma or CT26 colon carcinoma, induced specific resistance to a tumor challenge, and reduced the prevalence of lung metastases.

3.2 DaRT in combination with the immunomodulators CpG, cyclophosphamide (Treg inhibitor), and sildenafil (MDSC inhibitor), increased the cure rate.

3.3 DaRT and the immunoadjuvant, poly (I:C) retarded the growth of Triple-negative breast cancer (4T1), pancreatic (Panc02), and squamous cell carcinoma (SQ2)-derived tumors and elicited specific anti-tumor activity.

3.4 DaRT and poly (I:C) treatments in combination with cyclophosphamide or the epigenetic drug, decitabine or the checkpoint inhibitor, anti PD-1, intensified the effects of DaRT + adjuvant and promoted lung metastasis clearance.

4. Feasibility and safety of DaRT was examined in cancer patients with squamous cell carcinomas of the skin and head and neck. Cancer patients (ages 61-102) were treated for two weeks with DaRT (2 μCi Ra-224/seed, placed 5 mm apart). Complete response (CR) was observed in 22 lesions (22/28; 79%); 6 lesions (6/28, 21%) manifested a partial response. Among the 22 lesions with a CR, 9 developed a subsequent local relapse at the site of DaRT implantation at a median time of 6.2 months (range: 2.8-35.4 months). The 2-year overall survival rates post-DaRT implantation was 65% (95% CI: 42%-81%) among all patients and was 77% (95% CI: 50%-91%) among complete responders. In one patient, DaRT treated skin lesion disappeared after 76 days, and two other non-treated distant lesions also disappeared, probably due to an immune-mediated response.

Conclusions: Intra-tumoral DaRT, offers a technique for local tumor destruction, regardless of its replication status or hypoxic condition, and in addition to eliminate local and distant malignant cells, by immune-response-dependent mechanisms. DaRT is currently the only efficient method for treatment of solid tumors by alpha radiation. This combined treatment modality holds significant potential for the treatment of non-resectable human cancers.

Multiplexed ImmunoSpot® assays enable detailed assessment of antigen-specific B cell frequency, class usage and functional affinity

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Memory B cells constitute a critical component of humoral defense and are efficiently recruited into secondary immune responses upon antigen re-encounter. Expansion and subsequent differentiation of memory B cells into antibody-secreting cells (ASC) serves to rapidly increase antibody levels and limit dissemination of infectious agents. Moreover, in comparison to the short half-life of secreted antibody and its eventual decline in the absence of continuous replenishment, memory B cells are long-lived and thus serve as more reliable indicators of past antigen exposure(s). Presently there are few techniques that enable reliable identification of antigen-specific B cells at single-cell resolution. Unlike flow cytometric approaches, which depend upon surface B cell receptor-mediated acquisition of fluorescently-conjugated probes, ImmunoSpot® permits identification of rare antigen-specific B cells on the basis of secretory foot-prints generated by individual ASC. To highlight the utility of multiplexed and inverted FluoroSpot for detailed assessment of underlying B cell immunity, we characterized memory B cell-derived ASC reactivity against antigens representing SARS-CoV-2, seasonal influenza and Epstein-Barr virus (EBV) following polyclonal stimulation of cryopreserved peripheral blood mononuclear cells (PBMC) from convalescent COVID-19 donors. Elevated frequencies of ASC with reactivity against SARS-CoV-2 Spike and Nucleocapsid were readily detectable in convalescent COVID-19 donors despite low levels of serum IgG reactivity, although their relative frequencies were variable. Multiplexed FluoroSpot also revealed that SARS-CoV-2-, seasonal influenza- and EBV-reactive ASC exhibited biased IgG subclass usage and predominantly secreted IgG1. Inverted FluoroSpot assays recapitulated both ASC reactivity and their relative precursor frequency, and also enabled assessment of functional affinity through titration of soluble SARS-CoV-2 antigen probes. Collectively, our data illustrate the utility of ImmunoSpot® for detailed assessment of memory B cell reactivity and the requirement for cellular immune monitoring efforts to reveal what serum antibody may not.

Cytokine-induced killer cells – new options for anticancer immunotherapy

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Cellular immunotherapy reached the oncological patient many years ago, but it is relatively new to Lithuania, where it's been less than a decade since the first licensed advanced therapy medicinal product (ATMP) manufacturing site was opened. Cytokine-induced killer T-cells (CIKs) are not antigen-bound, they can be manufactured from autologous, allogeneic or cord blood, and current technology allows for relatively rapid cell expansion, all of which makes CIKs very attractive cells for the patient, manufacturer and researcher alike. They are defined by the $CD3^{+}CD16^{+}$ phenotype, which differentiates them from the NK cells, and this is where most of the publications stop at. Here, however, we attempt to characterize those cells in more detail, expanding on the phenotype and performing a series of cytotoxicity assays. We undertook the effort to establish several manufacturing protocols in the G-Rex membrane bioreactor setup and compared their growth efficiency and therapeutic potency. Our cytotoxicity testing platform consists not only of the K562 cell line, which is the golden standard for NK activity testing, but we also included several breast cancer cell lines of various subtypes (luminal, triple negative) and different resistance to chemotherapeutics (doxorubicin, cisplatin). The majority of research is performed on healthy adult donor blood, but we also show a glimpse of results achieved with cord blood, as well as insights into a novel combination treatment involving *B.subtilis* lectin as pre-treatment before the traditional CIK therapy.

Regulation of natural killer cell responses in solid tumors

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Due to their ability to eliminate tumor cells, natural killer (NK) cells are increasingly used in cancer immunotherapy. While several studies have reported therapeutic benefit of NK cell infusion in patients with acute myeloid leukemia and myelodysplastic syndrome, infusion of NK cells has yet to yield beneficial clinical responses in patients with solid tumors. Given the immune suppressive nature of the tumor microenvironment (TME), tumor-infiltrating NK cells often display impaired functions. Furthermore, emerging data suggests that various cellular interactions and soluble factors within the TME can influence NK cells to assume immune regulatory properties. We recently identified a subset of tumor-infiltrating NK cells that suppress anti-tumor T cell responses via the production of IL-10 and TGFβ. Here, we provide further analysis of these tumor-infiltrating NK cells suggesting their potency to influence the TME via the crosstalk with myeloid cells, impact epithelial–mesenchymal transition to promote metastatic dissemination, and to interfere with immune checkpoint therapy.

Checkpoint inhibitors in metastatic melanoma patients: clinical study report

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Introduction: Primary cutaneous melanoma is one of the most dangerous malignant neoplasms that occur in a human organism. Though intense research for the effective medication continues, overall survival rates stay unsatisfying. Most cases of melanoma are found at early stages. However, a certain group of patients already have metastases during diagnostics or between stages of treatment. The most common anatomical zones of metastasis are distant skin and subcutaneous tissue, lymph nodes, lungs, liver, bones and brain. Until recently melanoma was considered to be one of the most resistant tumors for the traditional therapy including chemotherapy, radiotherapy and biotherapy with α2-Interferon (IFNα2). The treatment strategy for metastatic melanoma has changed greatly with appearance of target therapy and immune checkpoint inhibitors.

Primary goal: To evaluate the clinical effectiveness of metastatic melanoma treatment using different systemic therapy options.

Materials and Methods: 164 patients with metastatic melanoma were examined (78 men and 87 women with average age of 56,4 years) during treatment in Rivne Antitumor center between 2010 and 2022. The manifestation type was as it follows: 41 case – lung metastases, 27 – liver metastases, 8 – brain metastases, 29 – metastases in bones, 28 – combined visceral metastases, 11 both visceral metastases and bone invasion, 20 – vast invasion in soft tissue and other organs. By the type of therapy all cases were divided into three groups: basic chemotherapy (monotherapy and combined chemotherapy, n=61), biochemotherapy (chemotherapy with IFNα2, n=57) and immunotherapy with checkpoint inhibitors (PD-1 checkpoint inhibitors, n=46). The effectiveness of treatment was evaluated by indicators of 12-36 months progression-free survival and overall survival.

Results: Compared with monotherapy with one medical drug, the combination of several chemotherapeutic agents did not lead to significantly better survival (overall survival 8.6/8.8month, relative risk (RR) 0.536; 95% confidence interval (CI) 0.076–4.874; p=0.542); progression-free survival (3.5/4.1 month; RR 1.132; 95% CI 0.084–6.874; p=0.929). In comparison with chemotherapy, biochemotherapy improved progression-free survival (RR 0.159; 95% CI 0.021–0.632; p=0.006), although it did not affect significantly overall survival (RR 1.916; 95% CI 0.586–6.912; p=0.283). At the same time, it is worth mentioning that biochemotherapy had significantly higher toxicity indicators (p=0.025). As for immune checkpoint inhibitors, compared to chemotherapy, their use improved overall survival (RR 3.332; 95% CI 1.097–11.614; p=0.033) and affected progression-free survival rates (RR 2.590; 95% 1.093–6.340; p=0.030). The use of monoclonal antibodies to PD1 had a lower manifestation of toxicity than chemotherapy (p=0.032). Compared to biochemotherapy, the use of immune checkpoint inhibitors did not have a significant advantage in terms of progression-free survival (RR 1.319; 95% CI 0.51–3.358; p=0.55) but provided better overall survival rates (RR 1.903; 95% CI 1.097–5.4644; p=0.048) and better outcomes for toxicity assessment (p=0.012).

Conclusion: Use of immune checkpoint inhibitors for the treatment of metastatic cutaneous melanoma provides better therapy efficiency, than chemotherapy, and ensures longer life span and quality of life, in comparison with the use of biochemotherapy. Future research should be aimed at granting a more long-term effect on overall survival rates for patients with metastatic melanoma.

Insights into the immune subtyping of ovarian tumors

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The current tumor immunology paradigm emphasizes the role of the immune tumor microenvironment and distinguishes several histologically and transcriptionally different immune tumor subtypes. However, the experimental validation of such classification is so far limited to selected cancer types. We aimed to explore the existence of inflamed, excluded, and desert immune subtypes in ovarian cancer, as well as investigate their association with the disease outcome. We used the publicly available ovarian cancer dataset from The Cancer Genome Atlas for developing subtype assignment algorithm, which was next verified in a cohort of 32 real-world patients of a known tumor subtype. Using clinical and gene expression data of 489 ovarian cancer patients in the publicly available dataset, we identified three transcriptionally distinct clusters, representing inflamed, excluded, and desert subtypes. We developed a two-step subtyping algorithm with COL5A2 serving as a marker for separating excluded tumors, and CD2, TAP1, and ICOS for distinguishing between inflamed and desert tumors. The accuracy of gene expression-based subtyping algorithm in a real-world cohort was 75%. Additionally, we confirmed that patients bearing inflamed tumors are more likely to survive longer. We also observed the differential circulating chemokine profile among the subtypes, which could be also explored further as representative systemic biomarkers. Moreover, when we applied a similar subtyping algorithm to melanoma tumors, COL5A2 gene also appeared as a top gene for separate the excluded subtype, and was able, together with IRF1, to distinguish three immune subtypes with 75%-95% accuracy in different validation cohorts. Our results highlight the presence of transcriptionally and histologically distinct immune subtypes among ovarian tumors and emphasize the potential benefit of immune subtyping as a clinical tool for treatment tailoring.

Genetically modified T cells for the immunotherapy of cancer

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Adoptive transfer of TCR and CAR gene modified T cells to treat cancer patients has been shown to have therapeutic efficacy. While some patients respond to therapy and have favorable outcomes, many progress despite T cells expressing a high affinity TCR or CAR. We have now treated a total of 28 patients with TCR or CAR gene modified T cells. Based on our preclinical and clinical studies, we find there are many factors that influence target recognition by TCR or gene modified T cells. Factors such as TCR affinity, transgene expression, and T cell physiology are critical features for effective cancer immunotherapy.

Moving from Hematologic Malignancy to Solid Tumors: Pathways to Effective CAR-T Therapy for Pediatric Solid Tumors

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The first approved CAR-T cell product was for pediatric pre-B ALL. The rapid responses seen reflect the biology of the disease, with non-transformed B cells and leukemia cells driving responses. In pediatric solid tumors, the physiological context suppresses CAR-T activity, as in other solid tumor indications. By optimizing the CAR-T cell product and subverting myeloid cell-induced immunosuppression, avenues to develop new therapeutic advances for solid tumors are being made.

Tumor-induced immune suppression: The problem is RAGE

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The development, expansion, and function of myeloid-derived suppressor cells (MDSC) has been attributed to multiple ligands and receptors. Most of these mechanisms are associated with inflammation that accompanies the microenvironment of many solid tumors, bacterial and viral infections, obesity, sepsis, and autoimmunity, among other conditions. MDSC are present at varying levels in virtually all individuals with cancer, where they inhibit the development of effective antitumor immunity. The multitude of known ligands that drive MDSC is making it challenging to develop strategies and drugs that specifically target, eliminate, or inactivate MDSC. Multiple inflammatory diseases are attributed to activation through The Receptor for Advanced Glycation Endproducts (RAGE) and signaling through RAGE activates many of the pro-inflammatory mediators that are established activators of MDSC. Using in vivo and in vitro approaches and genetically modified mice, our recent studies in conjunction with previous studies suggest that RAGE may be a key receptor on myeloid cells for driving the differentiation, accumulation and function of MDSC.

Peripheral blood biomarkers predicting clinical response to checkpoint blockade in melanoma

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Many years ago, we hypothesized that melanoma patients possessing T cells reacting to cancer antigens would do better than those who did not. To test this, we cultured PBMC from conventionally-treated metastatic melanoma patients with short (6 month) vs. long (18 month) survival with mixtures of peptides representing the shared cancer antigens NY-ESO-1, Melan-A, MAGE-A3 and survivin. After 12 days, cultures were restimulated with the same antigens and 24 h later intracellular cytokines measured by flow cytometry. The results were consistent with there being a clinical benefit of possessing T cells reactive to NY-ESO-1 and Melan-A but not MAGE-A3 or survivin, and patients possessing T cells reactive to both NY-ESO-1 and Melan-A did better than those with T cells responding to either alone. A dissection of the nature of the responding cells indicated that the majority of T cells responding to NY-ESO-1 was CD4+ whereas the majority responding to Melan-A was CD8+. Responding cells producing predominantly proinflammatory cytokines (IFN, TNF) were associated with clinical benefit, whereas IL 4 production was associated with poorer survival. Following these results, recent studies have focussed on whether similar findings apply to stage IV melanoma patients treated with anti-PD-1 antibodies, or a combination of anti-PD-1 and anti-CTLA-4 antibodies. Patients´ PBMCs were tested before the start of checkpoint blockade (baseline, BL) and at a mean of 44 days during ICB (follow-up, FU). In the discovery cohort from 3 clinical centers (n=92) and a validation cohort (n=49), it was confirmed that the presence of NY-ESO-1 and Melan-A-reactive T cells at BL before ICB conferred clinical benefit, both in terms of PFS and OS. Intriguingly, it was further shown that a decrease of these reactive cells at day 44 FU was associated with better survival than either their retention in the blood or their de novo appearance. This was interpreted to suggest sequestration of the reactive cells in the tumour, for which some limited evidence has accrued. By combining such analyses with conventional blood biomarkers (of which primarily BL and FU lactate dehydrogenase and myeloid-derived suppressor cells were associated with clinical outcome), we may approach more closely to the aim of providing robust individualized immune monitoring methods informative for individual patient outcomes.

Myeloid cells and their reprogramming in cancer patient profiling and cure

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Diseases associated with chronic immune stimulation, including cancer, are often paralleled by the occurrence of the so called *'emergency myelopoiesis*' (EM). This is a tightly regulated process tilting bone marrow hematopoiesis toward the myeloid lineage in response to inflammatory mediators. EM is a host para-physiological response to maintain immune homeostasis through the release of regulatory myeloid cells globally defined as *myeloid-derived suppressor cells (MDSC)*. MDSC expansion in peripheral circulation is a frequent finding in cancer patients and represents a constant biomarker of poor prognosis and treatment resistance across malignancies. This link is due to the immunosuppressive and protumor properties that EM-driven myeloid cells mediate at systemic and tumor level as detrimental consequence of their homeostatic function.

The main focus of our research activity is to comprehensively investigate cancer-associated EM in human setting, with the global aim of defining prognostic and predictive biomarkers as well as novel EM-related therapeutic targets. A *deep MDSC phenotypic profiling* by single cell flow cytometry, performed in different cancer clinical setting, is unravelling the complexity and heterogeneity of this population, but also the common traits for MDSC quantification in real-life clinical practice (Huber et al., JITC 2021).

The development of ex-vivo human MDSC models has allowed to generate *specific RNA and miRNA signatures* as prognostic biomarkers that predict poor clinical outcome and resistance to immune checkpoint inhibitors (Rinchai et al., *Clin Transl Med* 2021; Huber et al., *J Clin Invest*), and to intercept the myeloid-conditioning activity of antiangiogenics (Verzoni et al, *Annals Oncol* 2017; Verzoni et al., *Annals Oncol* 2018). MDSC-miRNAs have been also embedded into PLGA nanoparticles to generate an off-the-shelf tool for the fine tuning of myeloid cells and EM in different clinical settings.

Finally, a marked systemic MDSC rewiring, paired to enhanced memory and effector T cells in blood and at tumor site, could be observed after one single cycle of fasting-mimicking diet or moderate physical exercise in melanoma and breast cancer patients (Vernieri et al., *Cancer Discovery* 2022); this evidence proves the role of host metabolic conditions in EM and the impact of *short-term life-style changes* on MDSC frequency and function.

Altogether, our data point to EM as a key process in tumor immunity control and a rich source or biomarkers as well as therapeutic targets that should be exploited to improve cancer cure by comprehensive approaches of immunomodulation.

Immunological essentials for optimizing the clinical effect of whole tumor cell-based vaccines

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Immunotherapy became a useful alternative for metastatic diseases. Particularly, those based on immune checkpoint blockers showed significant success in promoting tumor regression and prolonging patient survival. However, many patients do not respond or develop resistance, making it necessary to explore combinatorial therapies to enhance adaptive responses, where cancer vaccines may constitute an attractive strategy. Different kinds of vaccine formulations have been tested, including those based on recombinant antigenic polypeptides or proteins, dendritic cells loaded with tumor-associated antigens (TAA), or RNA-DNA vaccines, with dissimilar success. Evidence showed that cancer vaccines, to be effective, must meet essential criteria such as; optimal delivery of a broad battery of TAA, presence of tumor biological agents acting as generators of danger signals, and combination with adequate adjuvants. These promote an early robust local inflammation and trigger adaptive immunological responses that reflect in a proinflammatory tumor environment capable of controlling tumor growth as our results shown. Whole-tumor cell-based vaccines have been extensively tested in various formats, either as intact or genetically modified cells or as tumor lysates together with various adjuvants. Our own and others' experiences, with strengths and weaknesses, will be reviewed here in order to establish the essential elements required to increase vaccine immunogenicity and clinical success.

Salmonella Typhi ZH9 as a novel microbial immunotherapy for bladder cancer

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Microbial immunotherapy of cancer has an over a century long history. The inflammation caused by bacteria can cause tumor damage and induce different magnitudes of immune response, including anti-tumor immune responses. However, to date there is only one approval in the field, namely Bacillus Calmette-Guerin (BCG) in non-muscle invasive bladder (NMIBC). Recent advances in understanding of immunological processes and gene-editing technologies warrant renewed efforts in advancing the microbial immunotherapy field.

40-80% of BCG treated patients experience disease progression within 5 years, ultimately requiring surgical bladder removal (cystectomy) which is associated with increased morbidity and a reduced quality of life. There is also a high risk of progression to metastatic disease (50%), particularly if surgery is not possible. Despite having high initial efficacy and being the standard of care for 30 years, BCG therapy has several further shortcomings, including complex legacy manufacturing and a prolonged dosing schedule comprising 6 weekly intravesical instillations to get maximal effect and up to 27 maintenance doses over the course of 3 years. Thus, there is a need and space for novel therapeutic approaches in NMIBC management.

Here we present the live attenuated *Salmonella enterica* serovar Typhi strain ZH9 (ZH9), as a novel microbial immunotherapy for NMIBC treatment. Leveraging the invasive, tumor-homing nature of S. Typhi whilst abrogating its ability for systemic spread provides a powerful new platform for cancer immunotherapy.

Our preclinical data demonstrate that intravesical ZH9 treatment induces activation of local innate as well as adaptive immunity in the murine bladder, resulting in accumulation of dendritic cells, T cells and NK cells. Furthermore, single intravesical ZH9 installation, but not BCG, results in significantly improved survival and also protects cured animals from repeated tumor engraftment in the orthotopic MB49 tumor model.

Recent data suggest that intradermal BCG immunisation can improve clinical outcome of BCG NMIBC treatment. Hence, we investigated the effect of systemic priming with ZH9 prior to intravesical ZH9 treatment and observed further improvement of survival. This finding suggests that the addition of a ZH9 prime to the clinical treatment schedule can warrant a reduced frequency of intravesical dosing without the loss of treatment effectiveness.

Based on our preclinical findings and given the extensive safety record of orally administered ZH9 in several vaccine clinical trials, this program is preparing to enter clinical evaluation in NMIBC patients. In addition to the primary objective of determining the safety and tolerability of ZH9 administered intravesically following oral priming with ZH9 in patients with NMIBC, we will conduct an extensive translational and biomarker investigational package to explore the mechanism of action of ZH9 and the type and magnitude of local and systemic immune responses.

Immunoregulatory neuroglial cells in cancer

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Tumor progression and expansion depends on the crosstalk between the malignant cells and stromal microenvironmental elements including fibroblasts, immune and endothelial cells. Neuronal components of the peripheral nervous system are also present in solid tumors, although their role in tumor development and spreading remains incompletely understood. Our data suggest that the Schwann cells, the main glial cells of the peripheral nervous system covering neurons dendrites and axons, are identified in human and mouse solid tumors and involved in the formation of unique tumor neuroenvironment. Schwann cells can directly stimulate tumor cell motility and spreading and can attract and modulate activity of immune regulatory cells – DC, MDSC and Treg cells. Using human and murine model systems, we have revealed that the interaction between cancerous cells, peripheral neurons and neuroglial cells plays an unanticipated role in tumor growth and formation of metastasis *in vivo*. Our data revealed that neuroglial cells can be activated by tumor cells resulting in up-regulated release of chemokines, cytokines and microRNA and thus attraction and activation of immune regulatory cells that form specific inflammatory-like immunosuppressive microenvironment. Several tumor-derived and glia-derived factors responsible for polarization of immune cells in the tumor milieu have been identified. Together, our new data (i) introduce new players in the tumor milieu and (ii) present a new concept of the tumor-neuronal-immune axis controlling activity of immune and malignant cells during tumor progression. These data will lead to the development of a novel neuroglia-centric antitumor therapeutic strategy: mechanism-based targeting of neuroglial elements in the tumor microenvironment.

Bifidobacterium animalis live cells and their metabolites suppress growth of breast cancer cells *in vitro*.

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Modern genetic methods of research significantly renewed interest in the field of human microbiota and its impact on human organism in health and pathological conditions. It was shown that microbiota plays a great role in the development and progression of human tumors. Microbiota also has a great impact on the efficacy of chemotherapy, radiotherapy and especially immunotherapy of cancer patients. The influence of microbiota on breast cancer was evidenced in many contemporary investigations. This impact could be direct and non-direct through manipulating activity of the immune, metabolic and hormonal systems. The representatives of human microbiota could demonstrate either tumor stimulating or antitumor activity. The interaction of bacteria with tumor cells is complex and multifactorial.

Bifidobacteria are known to have antitumor activity. This bacteria could display both direct contact-dependent and distant metabolite-associated mechanisms of action on tumor cells. Also, Bifidobacteria are known to influence activity of the immune system. The molecular details of the mechanism of interaction between probiotic bacteria and tumor cells are now actively studied.

We investigated the influence of live and heat inactivated Bifidobacteria and their metabolites on breast cancer cell lines of different molecular subtypes: T47D ($p53^{mut}$, ER+, HER-2/neu-), MCF-7 ($p53^{wt}$, ER+, HER-2/neu-) and MDA-MB-231 ($p53^{mut}$, ER-, HER-2/neu-). The cell insert cocultivation system for tumor cells and live Bifidobacteria was used. We studied the survival of tumor cells after the treatment with *Bifidobacterium animalis* live cells, heat-inactivated bacteria and bacterial metabolites at different tumor cell/bacterial cell ratios. Levels of p53 and bcl-2 expression were estimated by immunohistochemistry staining. Reactive oxygen (ROS) and cardiolipin (CL) content in tumor cells were analyzed by flow cytometry.

Baseline expression levels of proteins as well as cardiolipin and ROS varied in breast cancer cells of different molecular subtypes. Cells of each tumor cell line had their own specific biochemical profile of response to incubation with live Bifidobacteria and Bifidobacteria metabolites. It should also be noted that the reactions of tumor cells to incubation with live bacteria and their metabolites also varied. We thus found that Bifidobacteria and their metabolites influenced biochemical background and suppressed the growth of breast tumor cells lines T47D, MCF-7 and MDA-MB-231.

The exercise of TAMing the immune system

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Cells of the immune system, e.g., CD8 T cells and Natural killer (NK) cells are capable of recognizing and killing cancer cells. Naturally elicited anti-cancer immune responses lead to infiltration of immune cells into tumors and the presence of these cells impact on disease progression, i.e., overall survival of the patient and also correlate with response to check point inhibition (CPI) therapy. We recently demonstrated that tumors in exercising mice show increased immune cell infiltration, in turn associated with an over 60% reduction in tumor incidence and growth across several murine tumor models. Mechanistically we could show that efficacy was dependent on NK cells, which were engaged through an epinephrine-dependent mobilization. Together these results link exercise with improved immunological control of tumor progression, suggesting that exercise could be a beneficial partner for immunotherapy. We have now initiated a clinical trial where exercise is combined with immunotherapy (NCT04263467).

Numerous receptor-ligand interactions impact on T cell function in the tumor micro-environment (TME). We have characterized a novel co-stimulatory pathway in CD8 T cells, which goes via activation induced surface expression of both the receptor (MerTK) as well as the soluble ligand (Pros1). Importantly, increased signaling via this pathway leads to increased cytokine release, proliferation, and cancer cell killing. Interestingly, cancer cells as well as cells of the innate immune system, e.g., macrophages express the same receptor on the surface which implies that cancer cells may hijack the ligand and thereby convert a co-stimulatory signal for the T cells into oncogenic signaling in the cancer cells.

Immunoediting of acute leukemia by aberrant splicing

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Our most recent work informed the central hypothesis that non-canonical exon usage plays a dual role in leukemia and other pediatric cancers. On the one hand, it provides cancers with intrinsic mechanisms of epitope loss, which can render targeted immunotherapy ineffective. On the other hand, alternative splicing could be a source of cancer-specific epitopes and as such could aid immunotherapy. By simultaneously exploring the effects of alternative splicing on antigen loss and neo-epitope gain, we aspire to lay ground for the development of new immunotherapeutics that would target pediatric cancers with the specificity current modalities do not possess.

Prognostic and predictive value of circulating T lymphocytes in pancreatic ductal adenocarcinoma

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Background: Pancreatic ductal adenocarcinoma (PDAC) is highly fatal, especially in the third and fourth stages, where the 5-year survival rate does not exceed 10%. In the early stages, surgery is preferred, and advanced cancer is commonly treated with chemotherapy combinations. Although immunotherapy is being studied, it has not yet shown reliable clinical value. Furthermore, predictive and prognostic factors for choosing a more appropriate treatment strategy are still lacking.

Methods: Our study aimed to evaluate how surgery and chemotherapy change immune system parameters and whether these changes influence survival outcomes. We sought to identify an easily accessible marker to help choose the appropriate treatment. Patients with early PDAC were treated with surgery and adjuvant chemotherapy, and in later-stage chemotherapy, a combination of mFOLFIRINOX or gemcitabine monotherapy was used. Peripheral blood samples were obtained at baseline and after two and four months of treatment. Lymphocyte subsets were measured using flow cytometry. Correlation with clinical features and survival analyses were performed. In total, 196 patients were enrolled in this study. Eighty-eight patients underwent surgery, and 106 systemic therapy only. Then analyzing the whole study population, lymphocyte count, CD3+ cells, CD3+CD4+, CD3+CD4-CD8- subpopulation count correlated with cancer stage and CD4+CD25+ decreased through age.

Results: Forty-one patients' samples were available for changes over time analysis, and a decline in CD19+ B lymphocytes, natural killer (NK) cells CD3-CD56+CD16+, and T regulatory cells CD4+-FOXP3+ during treatment was observed. NKT-like cells CD3+CD56+ and T cells CD3+CD8+ tended to increase after two months and decrease after that. Independent predictors of PFS and OS in operable PDAC cohort included CD8+CD25+CD127+/- T regulatory lymphocytes and clinical features: resection margin, lymph node status, and differentiation. In advanced populations, baseline CD8+CD57- T lymphocyte count demonstrated a clear independent impact on progression-free survival and OS. Gemcitabine showed better survival in patients with extremely low baseline CD8+CD57- levels.

Conclusions: Our data demonstrates circulating CD8+CD25+CD127+/-, CD3+CD8+ and CD8+CD57- T cell subpopulations measured before treatment in PDAC may be considered prognostic and predictive biomarkers.

MDSC generation and their targeting in melanoma

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Myeloid-derived suppressor cells (MDSC) represent a heterogeneous myeloid cell population that is accumulated and activated in tumor microenvironment under chronic inflammatory conditions and substantially contributes to immunosuppression, representing thereby a valuable therapeutic target. It has been recently demonstrated that such MDSC enrichment could be mediated not only by a long-term production of soluble inflammatory factors but also by extracellular vesicles (EV) secreted by tumor cells. Importantly, EV contain a broad range of proteins, mRNA, microRNA and lipids and are considered as mediators of intercellular communication.

We analyzed polymorphonuclear (PMN) and monocytic (M) MDSC subsets regarding their immunosuppressive capacity and recruitment mechanisms in murine melanoma. The CXCR2/CXCL1 axis was identified as a mediator of PMN-MDSC migration. Inhibition of CXCR2 resulted in a decreased infiltration of tumors with PMN-MDSC and increased survival of melanoma bearing mice. Furthermore, adjuvant treatment of mice with resected tumors reduced the infiltration of pre-metastatic sites with PMN-MDSC and the occurrence of distant metastasis. The decrease in PMN-MDSC infiltration was accompanied by an increase in NK cells. Another possibility of MDSC targeting is based on the inhibition of the transcription factor STAT3, orchestrating MDSC accumulation and acquisition of immunosuppressive properties. The STAT3 inhibitor Napabucasin abrogated the capacity of murine MDSC to suppress T cell proliferation. It induced apoptosis in murine MDSC and significantly increased expression of molecules associated with antigen processing and presentation on these cells. Melanoma bearing mice treated with Napabucasin showed prolonged survival accompanied by a strong accumulation of tumor-infiltrating antigen-presenting cells and activation of CD8 and CD4 T cells. In melanoma patients, circulating M-MDSC strongly expressed activated STAT3 that was associated with a worse progression free survival (PFS), indicating the role of STAT3 as a promising therapeutic target in these patients and as a predictive marker for their clinical outcome.

CD163^{hi} tissue-resident macrophages as drivers for cancer immunotherapy resistance

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One of the major hurdles in the immunotherapy of cancer is the occurrence of therapy resistance. Efforts to target the underlying mechanisms with additional therapeutic strategies are limited and have thus far proven to be largely ineffective. In this study, two mouse models of primary or secondary resistance against vaccine-induced tumor regression were studied. We analyzed the tumor microenvironment during different stages of tumor outgrowth following immunotherapy using high-dimensional flow cytometry and transcriptomics. Comparison of the immune infiltrate of tumors during early and late regression revealed a rapid change in the function of tumor-rejecting towards tumor-promoting macrophages, characterized by the loss of iNOS, CD40 and MHC-II and a gain in Arg1, PD-L1 and SIRPα expression. In concert a rapid exhaustion of the tumor-infiltrating T cells was observed, characterized by a loss of CD49a and Eomes, and a gain in the checkpoint markers CTLA4, NKG2A, TIGIT and CD39. Perturbation studies resulted in the identification of a small but discernible subset of CD163^{hi} tissue-resident macrophages, with high expression of tumor-promoting macrophage markers and a functional M2 transcription profile, but not other macrophages, to be responsible. In-depth analyses revealed that they localize at the tumor periphery and are more resistant to Csf1 inhibition when compared to other macrophages. Specific depletion of these CD163^{hi} macrophages identified these cells as key modulators of immunotherapy resistance. By comparing the transcriptomic signature of these macrophages with other macrophages and by *in vivo* validation, we identified the degradation of heme through Heme oxygenase-1 as the underlying mechanism of immunotherapy resistance. Finally, the transcriptomic profile of CD163^{hi} macrophages is highly similar to the human immunosuppressive M2 macrophage population, indicating that they represent a target to improve immunotherapy efficacy.

Smoldering inflammation, an insidious support to the tumor microenvironment development

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Long lasting chronic inflammation induces progressive fibrosis of the involved tissues. Chronic inflammation, a leading factor in many pathological processes, can address cancer establishment and evolution. Local changes of immune activation inside a tissue, if maintained and supported by the environment, can induce structural remodeling. Reciprocally, collagen accumulation can affect the local immunity. We have shown that the colonization of germ-free (GF) mice colon by conventional mice (CV) intestinal microflora quickly modifies the local and systemic immunity. Contemporarily, it induces a fast remodeling of the collagen scaffold in the intestinal mucosa. Using a rat model of chronic colitis (dextran sodium sulphate – DSS – induced colitis) and of carcinogenesis (using azoxymethane – AOM - carcinogen for the colon) we have shown that, in both models, inflammation activates remodeling of the collagen scaffold organization even when the mucosa appears recovered from the acute induction. Multi-photon confocal microscopy of CV and GF animal mucosa resulted with higher complexity in structure in the CV rats (with microbiome). The immunological data suggest that the response to the microbiota presence elicit effective homeostatic regulation in the healthy CV rats, to avoiding inflammation and maintaining cytokine levels near the spontaneous production found in the GF animals. These conditions establish what we define as “inflammatory threshold” of the mucosa, allowing a range of tolerance to the continuous immune activation. The results also indicated that the collagen scaffold adapts to the immune microenvironment conditions, and quickly it can be altered if the immune threshold is overcome. The dis-balance between pro-inflammatory and regulatory signals, found even under apparently normal o reduced levels of microenvironmental cytokines, generates a smouldering inflammation still capable to damage the tissue structure. Moreover, in a mouse pancreatic cancer model, IL-17 expression resulted important to address the evolution of profibrotic collagen organization. Concluding, inflammatory-threshold changes producing smouldering inflammation predisposes to chronic inflammation and/or cancer niches establishment. Cytokine levels and collagen scaffold remodeling measured in the tissue can offer possibly new diagnostic markers.

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Tumor cell-associated IL-1α involvement in progression and metastasis of breast carcinoma in mice

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Breast cancer is the most common diagnosed malignancy and the second leading cause of female cancer-related death worldwide. An inflammatory tumor microenvironment supports tumor invasiveness and metastatic development. Interleukin-1α (IL-1α) is a pleiotropic cytokine that affects inflammatory and immune responses. It is a unique cytokine that can be found in the nucleus, cytoplasm, associated with the membrane or secreted. IL-1α is active in both its intracellular and secreted forms and works as an alarm molecule in the tumor microenvironment (TME). In this study, we assessed the effects of intracellular IL-1α on the development of triple negative breast cancer (TNBC) in mice. We used the CRISPR/Cas9 system to suppress IL-1α expression in 4T1 breast cancer cells. IL-1α KO clones were assessed *in vitro*. We observed that a deficiency of IL-1α in tumor cells results in reduced invasiveness, as was shown by colony formation and adhesion assays. A decrease in levels of many pro-inflammatory molecules, such as GM-CSF, IL-6 and some chemokines was observed in 4T1 breast cancer cells compared to control 4T1 cells. To assess the effects of tumor-related IL-1α on TNBC progression *in vivo*, we used orthotopical injection into the mammary fat pat. In comparison to injection of control 4T1 cells, injection of IL-1α KO 4T1 cells into BALB/c mice led to a significant decrease in local tumor growth, as well as fewer lung metastases. We assessed the mechanisms by which IL-1α from tumor origin affects the TME and observed a significant decrease in most pro-inflammatory molecules in tumors obtained from mice that were injected with IL-1α KO 4T1 clones. There was no difference in the number of CD11b+ cells in the TME in mice from either group, but we noted that in mice injected with control 4T1 cells, the prevalent cells in tumors were neutrophils or myeloid derived suppressor cells; whereas, in mice injected with IL-1α KO 4T1 cells, the recruitment of these cells was minimal. Indeed, in mice injected with IL-1α KO 4T1 cells, most CD11b+ cells were also CD11c+ and NKp46+ antigen presenting cells. In tumors from IL-1α KO 4T1 cells, there was increased infiltration of CD8+ cells. To confirm that immune cells are important in tumor regression of IL-1α KO clones, we performed experiments with immunodeficient NOID.SCID mice. In these mice, both control and IL-1α KO clones grow similarly. In conclusion, tumor-associated IL-1α is responsible for TNBC progression in mice and is involved in the interplay between immunosuppressive pro-inflammatory cells and anti-tumor antigen presenting cells.

Itaconic acid underpins hepatocyte lipid metabolism in non-alcoholic fatty liver disease

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Itaconate, the product of the decarboxylation of cis-aconitate, regulates numerous biological processes. We and others have revealed itaconate as a regulator of fatty acid beta-oxidation, generation of mitochondrial reactive oxygen species and the metabolic interplay between resident macrophages and tumors. We now show that itaconic acid is upregulated in human non-alcoholic steatohepatitis and a mouse model of non-alcoholic fatty liver disease. Mice deficient in the gene responsible for itaconate production (Immunoresponsive gene /Irg-1) have exacerbated lipid accumulation in the liver, glucose and insulin intolerance and mesenteric fat deposition. Treatment of mice with the itaconate derivative, 4-OI, reverses dyslipidemia associated with high fat diet feeding. Mechanistically, itaconate treatment of primary hepatocytes reduces lipid accumulation and increases their oxidative phosphorylation in a manner dependent upon fatty acid oxidation. We propose a model whereby macrophage-derived itaconate acts in trans upon hepatocytes to modulate the liver's ability to metabolize fatty acids. These findings suggest that interventions to alter itaconate levels may hold therapeutic potential to regulate metabolic disease and dyslipidemia.

Improving ACT therapy through combination with a DC tumor vaccine and protecting NK cells and T cells against oxidative stress

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Immunotherapy is increasingly used as a treatment modality across several different cancers. One category of immunotherapy, cell therapy, describes the process of infusing living cells, either via adoptive cell therapy (ACT) of T or NK cells or as a vaccine based on specialized antigen presenting cells, i.e., dendritic cells (DC). ACT in the form of tumor-infiltrating lymphocytes (TIL) has shown encouraging results in patients with metastatic melanoma. However, clinical effect of TIL therapy has so far not been proven in other solid tumors, including gynecological cancers. To further improve clinical responses in patients with melanoma we implemented a unique treatment strategy of TIL therapy combined with a DC-based cancer vaccine, clinical trial MAT02 (NCT01946373). The results are encouraging showing one patient with a strong partial response (PR, 6 years without disease progression), two patients with a complete response (CR, 5 and 3,5 years), and one patient with a mixed response (4/4 fully treated patient responding). We hypothesize that this combined cell therapy approach will also benefit patients with other solid tumors other than melanoma, with a focus on gynecological tumors.

Unfortunately, far from all patients are suitable for TIL production/therapy. Many tumors are inoperable or lack high T cell infiltration. As an alternative to TIL therapy, T cells derived from peripheral blood and stimulated ex vivo with tumor antigen pulsed DC are being applied in ACT. We have developed a novel strategy to deliver in silico predicted neoantigens to autologous dendritic cells (DC) using paramagnetic beads (EpiTCer beads). DC pulsed with EpiTCer beads are superior in enriching for healthy donor and patient blood derived tumor specific CD8+ T cells compared to DC loaded with whole-tumor lysate or neoantigen peptides.

Furthermore, the tumor microenvironment has been shown to be very hostile, including production of elevated levels of reactive oxygen species (ROS), which can impair NK- and T cell function. This represents a significant hurdle within the field of ACT which needs to be addressed to improve clinical responses. We have established a strategy to render human cytotoxic lymphocytes resistant towards oxidative stress. Pre-treatment of human NK cells and TIL ex vivo with a low dose of auranofin, an FDA approved Nrf-2 activating drug, significantly lowered their accumulation of intracellular ROS and preserved their antitumoral activity despite high H2O2 levels or ROS producing monocytes. Furthermore, auranofin pre-treatment of CD19 CAR-T cells or TIL increased their elimination of CD19+ tumor cells or autologous tumor spheroids, respectively, especially during ROS exposure. Analysis of Nrf2-driven target genes revealed that the increased resistance against ROS was Nrf2 dependent. These novel findings suggest that Nrf2 activation in human cytotoxic lymphocytes could be used to enhance the efficacy of adoptive cell therapy.

Microglia functions as a double-faced Janus in the progression of melanoma towards brain metastasis

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Cancer microenvironments contain both cancer promoting as well as cancer restraining factors. The balance between these opposing forces determines cancer demise, progression or dormancy.

Melanoma frequently metastasizes to brain. Once colonized in the brain, melanoma cells communicate with brain resident cells such as microglia, the resident macrophages of the CNS. The pathobiology associated with the interactions between brain-metastasizing melanoma cells and microglia is a major focus of our lab. An in-depth analysis of the molecular and functional consequences of the reciprocal signaling between human melanoma and microglia indicated that each cell type reprograms the phenotype, transcriptome, proteome and the miRNA landscape of the interacting partner. This mutual reprograming created a brain-metastasis-promoting vicious cycle.

'Microenvironmental control' was proposed by Eva and George Klein as a protective mechanism against cancer. According to this concept, the interaction of cancer cells with particular cells or molecules in the tumor microenvironment confers upon these cells growth arrest and/or death.

A previous study from our lab demonstrated that the beta subunit of human hemoglobin produced by endothelial kidney cells restrained the development of local neuroblastoma tumors as well as of lung and bone marrow metastasis. Recent findings (submitted for publication) indicated that factors derived from microglia restrain proliferation and induce apoptosis and necrosis of brain-metastasizing melanoma cells. Employing various purification procedures, we identified a heterodimer composed of hemoglobin alpha and beta chains that performs these anti-metastatic functions. An alpha/beta chain dimer chemically purified from human hemoglobin inhibited the cell viability of primary melanomas and of melanoma brain metastasis, induced DNA damage, cell cycle arrest at the SubG1 phase, apoptosis, and significant necrosis in melanoma brain metastatic cell lines. Proteomic analysis of dimer-treated melanoma cells revealed that the dimer downregulates the expression of several proteins playing crucial roles in cancer cell sustainability and progression.

All together, these results indicate that microglia cells play a yin/yang role in the progression of melanoma towards brain metastasis.

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CITIM-2023 ABSTRACTS

CONFERENCE PARTICIPANTS

Microglial JunB promotes the malignant phenotype of brain metastasizing melanoma cells

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The reciprocal signaling between cancer cells and cells in their microenvironment reprograms the phenotype of both interaction partners. Our lab focuses on the crosstalk between brain-metastasizing melanoma cells and brain cells such as astrocytes or microglia, and on the impact of these interactions on the formation of melanoma brain metastasis (MBM). Previous studies demonstrated that the crosstalk between melanoma and microglia reprogrammed the phenotype and molecular signature of both melanoma as well as microglia cells. One of the microglial molecules that were significantly upregulated by melanoma-microglia interactions was the transcription factor JunB. In this study we set out to elucidate the impact of JunB, if any, on the progression of brain-metastasizing melanoma cells towards frank metastasis. It was found that the JAK signaling pathway controlled the JunB up-regulation in microglia cells following interaction with melanoma cells and that the melanoma-secreted cytokine Leukemia Inhibitory Factor (LIF) mediated this upregulation. The impact of JunB on microglia phenotype was investigated by employing microglia variants that stably over-express JunB ($JunB^{hi}$) or exhibit downregulated levels of JunB ($JunB^{lo}$). The use of these microglia variants revealed that JunB decreased the migratory capacity and the phagocytic ability of microglia cells. At the molecular level JunB negatively regulated the expression of Iba-1 and CD150 (key regulator of leukocyte activation and differentiation and M2 marker) and positively regulated the expression of the immunosuppressive molecules *SOCS3* and PD-L1, as well as MMP-2, in microglia. Moreover, JunB regulated the proteome of microglia i.e., the expression of CD44, CD171, connexin and β-catenin. We also studied the impact of the JunB variants on MBM cells and showed that factors released from $JunB^{lo}$ microglia reduced the migration capacity as well as the viability of melanoma cells, demonstrating that microglial JunB exerts pro-metastatic activities. Transcriptome analysis of ($JunB^{lo}$-exposed MBM cells revealed that JunB controls the expression levels of *SOCS3*, *NQO1* and *ALDOC* in these melanoma cells. Altogether, these data demonstrate that microglial JunB is a determinant of melanoma progression and therefore may serve as a novel target in melanoma theranostics.

The biological relevance of tumor-associated neutrophils as critical targets for immunotherapy optimization

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Background: Neutrophils are elevated in the circulation of patients with several cancer types and this neutrophilia correlates with poor clinical outcome. Tumor-associated neutrophils negatively regulate antitumor immunity and remain a promising target for therapy. However, the functions and phenotypes of neutrophils especially in the context of immunotherapy are largely unclear. Here, we evaluated the biological relevance of tumor-associated neutrophils in a murine cancer model and examined their modulation in therapy response.

Method: We established the syngeneic B16 tumor model subcutaneously in the C57BL/6 mice. Using flow cytometry, immunofluorescence, ELISA, quantitative PCR together with in vivo and in vitro systems, we investigated the relevance of functional neutrophil-T cell interplay on tumor growth and control.

Results: We observed the expansion of neutrophils in the spleen and their mobilization in circulation and lymph nodes during tumor progression. Tumor-infiltrating neutrophils displayed high activation, a mature phenotype and evidence of immune suppression. Additionally, priming of naïve neutrophils in B16-tumor conditioned medium revealed the phenotypic modulation of neutrophils in vitro, suggestive of polarization towards the MDSC subtype. In effect, depletion of these immunosuppressive neutrophils in vivo or inhibition of their intra-tumoral recruitment controlled tumor growth while increasing T cell intra-tumoral infiltration and their effector phenotypes. Interestingly, modulating neutrophils in combination with immune checkpoint inhibition improved tumor response, accompanied by increased frequency of granzyme B+ and perforin+ CD8 T cells compared with monotherapy.

Conclusion: Targeting of T cell suppressive neutrophil phenotypes improves T cell functionality and augments the efficacy of immune checkpoint blockade therapy.

HSP90α induces immunosuppressive myeloid cells in melanoma via TLR4 signaling

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Cancer progression and metastasis formation involves modulation of the host immunity, in particular myeloid compartment. For that, tumor cells secrete extracellular vesicles (EV) and soluble factors that are taken up by myeloid cells leading to the generation of myeloid-derived suppressor cells (MDSC). MDSC are characterized by the strong suppressive capacity against T and NK cells and have been shown to be associated with poor prognosis and resistance to immunotherapy in melanoma. EV from murine and human melanoma cell lines were demonstrated to upregulate programmed cell death ligand 1 (PD-L1) on myeloid cells and thereby induce MDSC. This conversion was dependent on the toll-like receptor (TLR) signaling and the presence of heat-shock protein 90α (HSP90α) in EV. In the current study, we studied whether HSP90α can convert monocytes into MDSC as sole soluble factor.

We isolated CD14+ monocytes from peripheral blood of healthy donor's and treated with human recombinant HSP90α (rHSP90α) for 16, 24, 48 or 72 h. Expression of immunosuppressive markers was analyzed by flow cytometry, Western blot, and microarray assay. To assess immunosuppressive function of rHSP90α-treated monocytes, an inhibition of T cell proliferation assay was performed. The level of HSP90α in melanoma patients and correlated with phenotype of circulating monocytes and MDSC and with clinical outcome.

Monocytes were found to strongly upregulate PD-L1 after 16 h treatment with rHSP90α, while reactive oxygen species (ROS) and NO production, expression levels of adenosine producing ectoenzymes CD39 and CD73, arginase-1 was not change. Upregulation of PD-L1 could be abrogated by an anti-TLR4 antibody or TLR4 inhibitor Resatorvid and by an NF-κB inhibitor (Bay). Upon further incubation for 24h, we observed a downregulation of HLA-DR in rHSP90α-treated monocytes. Additionally, rHSP90α treatment led to an increased viability of monocytes and apoptosis prevention. Monocytes incubated for 24h with rHSP90α acquired capacity to inhibit T cell proliferation in TLR4-, PD-L1-, and IDO1-dependent manner. Higher plasma levels of HSP90α in melanoma patients positively correlated with PD-L1 expression on circulating monocytic MDSC. Moreover, melanoma patients receiving immune checkpoint inhibitors with higher levels of HSP90α displayed shorter progression-free survival.

Taken together, we demonstrated a TLR4-NF-κB-dependent mechanism of monocyte conversion into MDSC by soluble HSP90α, suggesting additional targets for inhibition of MDSC and overcoming immunotherapy resistance in melanoma.

The involvement of immune-related miRNAs in lung cancer

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Lung cancer is the leading cause of cancer death world-wide. Cancer-associated immune response, which is present at different stages of tumorigenesis, contributes heavily to cancer growth and metastasis. The early cancer diagnosis and control of cancer metastasis are critical factors in cancer treatment. Discoveries of novel biomarkers and therapeutic targets have helped to increase survival rates for many types of cancer, including lung cancer. Over the past decade miRNAs have emerged as potential biomarkers and targets in lung cancer diagnosis and treatment. miRNAs are small 20-25 nucleotide long single-stranded non-coding RNAs that play an important role in tumor initiation and growth. miRNAs function in post-transcriptional regulation of gene expression and can act as tumor suppressors, oncogenes or metastasis regulators.

The previous study at the National Cancer Institute, Vilnius, Lithuania identified a set of miRNAs that can be associated with lung cancer metastasis 1). A number of immune-related miRNAs were selected for further investigations into their expression signatures in normal and cancerous lung tissue biopsies with the aim to identify novel potential biomarkers for lung cancer.

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Definition of systemic immune suppression cut-off values in cancer patients for future clinical applications

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Background and Aims: Myeloid derived suppressor cells (MDSC) blood frequency is associated to therapy resistance (Wesolowski *et al.*) and is a relevant negative prognostic factor in different cancer settings. Recently, an in-lab developed myeloid index score (MIS), based on the frequency of circulating $CD15^+$, $CD14^+$, $CD14^+PD\text{-}L1^+$ and $CD14^+HLA\text{-}DR^-$, stratifies advanced melanoma patients according to the disease aggression (Huber *et al.*). However, normal values that could distinguish MDSC in healthy donors (HD) from cancer patients still remain a clinical need. Exploiting a multicentric observational study (SERPENTINE) we performed a comprehensive immune profiling of HD and cancer patients, including melanoma, head and neck cancer, urothelial cell carcinoma and renal cell carcinoma, with the purpose of developing an off-the-shelf assay and predictive tool across multiple cancer types.

Methods: HD (n=123) and cancer patients (n=43) enrolled in SERPENTINE trial underwent blood immune monitoring; peripheral blood samples were collected before the first treatment cycle (T0), before the second therapy cycle (T1), and at the time of disease revaluation (after 3 months, T2), with an additional blood collection in case of disease progression (PD). Immune profiling was performed by high-resolution flow cytometry using an antibody panel designed to detect M-MDSC and PMN-MDSC in whole blood (CD14, PD-L1, HLA-DR, CD15, CD33, CD10, CD16, LOX-1, CD45, CD8, and PD1). Cell absolute count (cells/µL) was obtained with flow count fluorospheres. All the enrolling centers among Europe (n=4) underwent to a harmonization process of the flow cytometers in order to standardize the FACS acquisitions.

Results: Flow cytometry data show a significant increase of circulating MDSC in cancer patient at T0, regardless of the tumor type, compared to HD. Specifically, $CD14^+HLA\text{-}DR^-$ and $CD14^+PD\text{-}L1^+$, which characterized M-MDSC subpopulations, were significantly higher in cancer patients compared to HD (16.8±11 *vs.* 5.5±3.1 of $CD14^+HLA\text{-}DR^-$, and 1.3±1.3 *vs.* 0.9±0.8 of $CD14^+PD\text{-}L1^+$, respectively). In parallel, PMN-MDSC (defined as $CD15^+LOX1^+$) were also higher in cancer patients than HD. In addition, we identified two granulocytic populations, as mature ($CD15^+CD16^+CD10^+LOX\text{-}1^+$) and immature ($CD15^+CD16^-CD10^-LOX\text{-}1^+$) subsets, whose frequency was remarkably increased in cancer patients. Further analyses have been done among HD subjects. In this population, sex did not affect the MDSC frequency, while age influenced the frequency of $CD14^+HLA\text{-}DR^-$ cells, showing an increase in the 32-44 group compared to the other age groups.

Conclusion: Encouraging preliminary results showed remarkable differences in the frequency of suppressive and inflammatory monocytes between cancer patients and healthy subjects, suggesting the possibility to define a cut-off value for MDSC quantification in whole blood of cancer patients. The assessment of the immunosuppression status could provide advantage in clinical practice as a tool for monitoring disease progression.

A heterodimer of α and β hemoglobin chains functions as an innate anticancer agent

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Metastatic (as well as tumor) microenvironments contain both cancer-promoting as well as cancer restraining factors. The balance between these opposing forces determines the fate of cancer cells that disseminate to secondary organ sites. In search for microenvironmental drivers or inhibitors of metastasis, we identified, in a previous study, the beta subunit of hemoglobin (HBB) as a lung-derived antimetastatic factor. In the present study, exploring mechanisms leading to melanoma brain metastasis, we discovered that brain-derived factors restrain proliferation and induce apoptosis and necrosis of brain-metastasizing melanoma cells. Employing various purification procedures, we identified a heterodimer composed of hemoglobin alpha and beta chains that perform these anti-metastatic functions. Neither the alpha nor the beta subunit alone was inhibitory. An alpha/beta chain dimer chemically purified from human hemoglobin inhibited the cell viability of primary melanomas, melanoma brain metastasis (MBM), and breast cancer cell lines. The dimer-induced DNA damage, cell cycle arrest at the SubG1 phase, apoptosis, and significant necrosis in four MBM cell lines. Proteomic analysis of dimer-treated MBM cells revealed that the dimer downregulates the expression of BRD4, GAB2, and IRS2 proteins, playing crucial roles in cancer cell sustainability and progression.

Nivolumab plus cabozantinib versus sunitinib for first-line treatment of advanced renal cell carcinoma: 3-year follow-up from the phase 3 CheckMate 9ER trial

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Background: First-line nivolumab plus cabozantinib (NIVO+CABO) demonstrated superiority over sunitinib (SUN) with 25.4 months minimum follow-up (median, 32.9 months) in patients with advanced renal cell carcinoma (aRCC) in the CheckMate 9ER trial. Here, we report survival, response per blinded independent central review (BICR), and safety after 3 years minimum follow-up in all randomized patients and by International Metastatic Renal Cell Carcinoma Database Consortium (IMDC) risk score.

Methods: Patients were randomized 1:1 (stratified by IMDC risk score, tumor PD-L1 expression, region) to NIVO 240 mg flat dose intravenously every 2 weeks plus CABO 40 mg once daily orally versus SUN 50 mg once daily orally for 4 weeks (6-week cycles) until disease progression or unacceptable toxicity (maximum NIVO treatment, 2 years). The primary endpoint was progression-free survival (PFS) by BICR. Secondary endpoints were overall survival (OS), objective response rate (ORR) by BICR, and safety.

Results: In total, 323 patients were randomized to NIVO+CABO and 328 to SUN. With 36.5 months minimum follow-up (median, 44.0 months), PFS and OS benefits were maintained with NIVO+CABO versus SUN in intent-to-treat patients. Median PFS was 16.6 versus 8.4 months (hazard ratio [HR] 0.59 [95% confidence interval [CI], 0.49–0.71], $P < 0.0001$) and median OS was 49.5 versus 35.5 months (HR 0.70 [95% CI, 0.56–0.87], $P = 0.0014$). ORR (95% CI) was higher with NIVO+CABO versus SUN (56% [50–62] vs 28% [23–33]), and 13% versus 5% of patients achieved complete response, respectively. Median duration of response was 22.1 versus 16.1 months for NIVO+CABO versus SUN. PFS, OS, and response are reported across prespecified IMDC risk groups in the Table. Any-grade treatment-related adverse events (TRAEs) occurred in 97% versus 93% of patients treated with NIVO+CABO versus SUN (grade ≥ 3 TRAE, 67% vs 55%). TRAEs led to discontinuation of CABO only in 10% of patients, NIVO only in 10% of patients, NIVO+CABO in 7% of patients, NIVO or CABO in 28% of patients, and SUN in 11% of patients.

Conclusions: After 3 years minimum follow-up, survival and response benefits were maintained with NIVO+CABO and remained consistent with previous follow-ups. Median OS with NIVO+CABO improved by 11.8 months since the previous data cut. Responses with NIVO+CABO were durable, with higher complete response rates with NIVO+CABO versus SUN regardless of IMDC risk group. No new safety signals emerged with additional follow-up in either arm. These results continue to support NIVO+CABO as a first-line treatment for patients with aRCC.

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	FAV NIVO+CABO (n = 74)	FAV SUN (n = 72)	INT NIVO+CABO (n = 188)	INT SUN (n = 188)	Poor NIVO+CABO (n = 61)	Poor SUN (n = 68)	I/P NIVO+CABO (n = 249)	I/P SUN (n = 256)
mPFS (95% CI), months	21.4 (13.1–24.7)	13.9 (9.6–16.7)	16.6 (11.9–20.0)	8.7 (7.0–10.4)	9.9 (5.9–17.7)	4.2 (2.9–5.6)	15.6 (11.2–19.2)	7.1 (5.7–8.9)
PFS HR (95% CI)	0.72 (0.49–1.05)	–	0.63 (0.49–0.80)	–	0.37 (0.24–0.57)	–	0.56 (0.46–0.69)	–
mOS (95% CI), months	NR (40.7–NE)	47.6 (43.6–NE)	49.5 (37.6–NE)	36.2 (25.7–46.0)	34.8 (21.4–NE)	10.5 (6.8–20.7)	49.5 (34.9–NE)	29.2 (23.7–36.0)
OS HR (95% CI)	1.07 (0.63–1.79)	–	0.75 (0.56–1.0)	–	0.46 (0.30–0.72)	–	0.65 (0.51–0.83)	–
ORR (95% CI), %	68 (56–78)	46 (34–58)	56 (49–64)	28 (21–35)	41 (29–54)	10 (4–20)	53 (46–59)	23 (18–29)
CR, %	16	10	15	4	5	1	12	4

CR, complete response; FAV, favorable; INT, intermediate; I/P, intermediate/poor; m, median; NE, not estimable; NR, not reached.

Effect of radiotherapy on DNA signaling cascades in head and neck cancer cells

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Introduction: Head and neck squamous cell carcinomas (HNSCC) are the most common type of cancer in the head and neck region. It has become obvious that radiotherapy (RT), besides its DNA damaging properties, has also immune modulatory potential. Further, latest research has shown that DNA damage and responses to it might also influence the immunogenicity of tumor cells. However, related immunogenic DNA sensing pathways consist of a heterogeneous group of molecules. We therefore aim to analyze which DNA sensing molecules in the cytoplasm of HNSCC cells following RT are prominent, and whether an inhibition of dominant sensing molecules impacts on cell death, immune checkpoint molecule expression and cytokine release.

Methods: Cell death induction by RT (19,3Gy and 5x3 Gy) on two HPV negative (Cal33, HSC4) and two HPV positive (UD2, UM47) HNSCC cell lines was analyzed by Annexin V-FITC/PI staining. Immune stimulatory and immune suppressive checkpoint molecules (PD-L1, PD-L2, OX40-L, ICOS-L, HVEM, CD134-L and CD70) were analyzed on the tumor cell surface by multicolor flow cytometry. Release of interferon-ß (INF-ß) as key cytokine for DNA sensing pathways in the supernatant was measured with an indirect enzyme-linked immunosorbent assay and mRNA expression of DNA sensing pathways (cGas/Sting, RIG-I) as well as immunostimulatory cytokines (INF-ß, IFIT1, IFIT3) was measured with polymerase chain reaction.

Results: Cell death induction mostly after 5x3Gy was similar in all of the examined HNSCC cell lines and consisted of a mixture of apoptosis and necrosis. But only for the HSC4 cell line, RT with 5x3Gy or 19,3Gy led to an increase in INF-ß in the supernatant as well as of INF-ß mRNA expression. The mRNA expression of RIG-I was increased in HSC4 cells under these conditions, too. The analyses of the same treatment schedules in combination with an inhibitor of the RIG-I pathway show a decrease in expression of immunostimulatory cytokines (IFIT1, IFIT3) as well as no upregulation of immunosuppressive checkpoint molecules (PD-L1, PD-L2) after radiation compared to the control group.

Conclusions: Not the cGas/Sting, but the RIG-I pathway plays the major role in the release of INF-ß and for an increased expression of interferon-stimulated genes in HSC4 cells. Also, the RIG-I pathway affects checkpoint expression. This pathway could be a promising candidate to be individually targeted in HNSCC, in order to alter the tumor immune microenvironment in favor of enhancing immunotherapy efficacy.

Colonic macrophages constrain T cell infiltrate through altered lipid metabolism

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Background: Among the major factor risks for CRC development, overweight and obesity play a crucial role, favoring cell hyper-proliferation and tumor growth. In this scenario, the impact of increased availability of lipidic effectors on immunosurveillance still is not well understood. Here, we investigated the role of lipid-engulfed colonic macrophages, also known as foam cells (FC), on adaptive immunity in early and late colorectal cancer (CRC).

Methods: Surgical specimens, including tissue lesions and unaffected mucosa, were collected from patients with colonic polyps, including (*n*=37) Familial Adenomatous Polyposis (FAP) and (*n*=58) FIT-positive subjects, together with (*n*=65) stage I-III CRC patients. Multiparametric flow cytometry and bulk RNA sequencing were performed to analyze the functional properties of the tissue immune infiltrate. Digital pathology was also applied to investigate the spatial distribution of FC versus T cell immunity. Obtained results were correlated with disease outcome and clinical parameters, including patients BMI and plasma lipid profile.

Results: A subset of large, $CD68^+$ macrophages, engulfed with neutral lipid droplets, were found within lamina propria, at the “root” of 45% hereditary and 25% sporadic polyps. FC were also present in 80% CRC, acquiring a “barrier-like distribution” at the tumor margin. FC presence was independent of most clinical factors but associated with high BMI, plasma saturated FAs. Within the tumor environment, FC inversely correlated with protective $CD8^+$ T cells, while positively associated with increased regulatory T cells (Treg). Most importantly, FC accumulation worsen diseases recurrence in a subset of CRC patients lacking protective $CD8^+$ immunity. In gut precancerous setting, FC accrual reflects an analogous immune microenvironment, which could be reverted by low-fat diet.

Conclusions: Lipid-laden colonic macrophage associate with immunosuppressed tumors and blunt $CD8^+$ effector-mediated cancer control. FC accrual in tumors with CD8-poor infiltrate identifies a subset of patients with high risk of recurrence after radical surgery for primary CRC. Being possibly modulated by circulating lipids of dietary origins and decreased in tissue after specific nutritional intervention, FC represent a promising target to enhance antitumor immunity and control CRC onset and progression.

Immunostimulatory properties of different length dsRNA and their use for anticancer dendritic cell vaccine adjuvants

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Background: Double-stranded RNA (dsRNA) of different lengths can activate distinct immune system components. Larifan, a mix of natural-origin dsRNA (50-5000 bp) and thus potential adjuvant in developing anti-cancer vaccines, was extracted from f2 bacteriophage-infected *E. coli* cells and chromatographically separated by length into fractions. We aimed to investigate the biological activity of dsRNA different fraction as an adjuvant and select the potential candidates for anti-cancer dendritic cell (DC) therapy.

Methods: We investigated the effect of Larifan fractions (FR) as an immunostimulatory agent (adjuvant) on the morphology of DCs, the expression of genes and surface markers, and the secretion of cytokines. The immunogenic (DECTIN1, IL-12, TNF-α, XCR1, ICOSL, CD209a, CCR7) and tolerogenic (IDO, PDL1, TGF-β) gene expression of DCs maturated with dsRNA fractions of different lengths was compared with the gene expression of DCs matured with LPS adjuvant (control group). The following dsRNA fractions were used for DCs maturation: long FR3 (500-1000 bp), medium-length FR9 (200-500 bp), short FR15 (50-200 bp) and unfractionated mixture dsRNA mix. DCs were differentiated from healthy C57BL/6 mice bone marrow. Gene expression was evaluated by PCR. The expression of DCs immunogenic surface markers CD40, CD80, CD86, MHCII, and CCR7 evaluated by flow cytometry. Cytokines IFN-γ and IL-10 secretion determined by ELISA.

Results: Evaluation of DCs gene expression showed that maturation of DCs with unfractionated dsRNA and FR15 was superior to maturation with other fractions due to lower expression of the tolerogenic Ido1 gene and higher expression of the immunogenic Clec7a, Tnf, Icosl, Il12rb2, Cd209a genes. The expression of DCs surface markers by flow cytometry showed that maturation with different dsRNA fractions resulted in high expression of all surface immunogenic CD40, CD80, CD86, MHCII, and CCR7 markers. ELISA analysis showed that maturation of DCs with different dsRNA fractions resulted in lower secretion of the immunosuppressive cytokine IL-10, compared to the standard maturation with LPS. No statistically significant differences in the immunostimulatory cytokine IFN-γ were found.

In conclusion *in vitro* studies have shown that dsRNA-activated DCs are functionally mature and able to initiate an anticancer immune response and are viable in immunotherapy, thus encouraging its future application.

Attenuated influenza viruses co-expressing bovine papillomavirus 1 (BPV1) antigens lead to tumour regression and eradication of BPV1/2 infection underlying equine sarcoid disease

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Papillomaviruses (PVs) are dsDNA viruses that can induce cutaneous or mucosal lesions ranging from benign warts to lethal cancers in humans and animals. Neoplastic transformation of cells is mediated by the viral oncoproteins E5, E6, and E7. In humans, carcinogenic high-risk PVs (hrHPV, e.g., HPV16, HPV18) are responsible for virtually 100% of cervical cancers. In horses and other equid species, closely related BPV1 and BPV2 commonly induce benign, yet locally aggressive skin tumours termed sarcoids that are highly therapy-resistant and tend to recur in a more progressive multiple form following accidental or iatrogenic trauma.

We have generated human influenza (Flu) A and B viruses deficient for the interferon-antagonist NS1 (dNS1) and co-expressing papillomavirus E6 and E7 antigens as innovative immunotherapeutic vaccine strategy. In mice with established HPV16-associated tumours, intralesional injection with dNS1 Flu A co-expressing mutated HPV16 E6E7 led to complete regression of lesions in 50%, and partial regression in 25% of animals. Based on these promising data, we created delNS1 Flu A and B viruses co-expressing shuffled BPV1 E6E7 peptides for sarcoid immunotherapy. Given that intradermal injection of healthy horses proved safe and induced a systemic immune response, the therapeutic potential of the vaccines was addressed in 29 horse patients affected by BPV1- (27) or BPV2-induced (2) mild (5), moderate (5) and severe sarcoid disease (19; multiple sarcoids of progressive type). Patients were closely monitored with respect to general well-being, sarcoid regression, and BPV1/2 infection status from the day of first intertumoral injection onwards. Sixty-two percent of patients responded to therapy, with 14 horses showing complete sarcoid regression (CR), and 4 horses exhibiting partial regression (PR) as of March 2023. Unsurprisingly, treatment was most efficacious in case of mild-type disease. However, CR was also achieved in one third of patients with severe disease. Poor response was mainly noted in horses bearing extensive lesions of verrucous type.

Importantly, BPV1/2-specific PCR from swabs and scraping collected from former tumour sites scored repeatedly negative in 9/10 patients with CR so far. In a current trial, immunotherapy is combined with surgery for treatment of severe disease: Intralesional injection of 1-3 sarcoids on day 0 is followed by surgical excision of all tumours on day 14. Intramuscular boosters are given on days 28 and 84. We monitored the infection status of the first patient (20+ sarcoids) by BPV1/2 PCR, revealing freedom from infection on day 56 (Fig. 1).

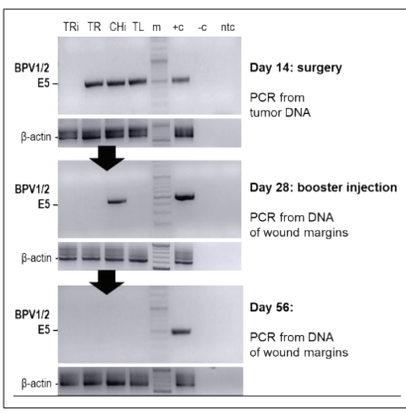

Figure 1: PCR monitoring of patient #1 following injection of two sarcoids with dNS1 Flu A-E6E7 on day 0.
TRi: sarcoid thorax, right side, injected
TR: sarcoid thorax right side
CHi: sarcoid chest, injected
TL: sarcoid thorax, left side
m: molecular weight marker
+c: sarcoid DNA (positive control
-c: equine normal skin DNA (negative control)
ntc: sterile water (no-template control.

Our data demonstrate that dNS1 Flu A/B viruses co-expressing shuffled BPV1 E6E7 peptides not only have the potential to cure sarcoid disease even in severely affected individuals, but also eradicate BPV1/2 infection. This latter finding is exceptional as the Flu-based immunotherapeutic is the first and only of its kind successfully targeting the viral infection underlying disease, and by this, minimizing the risk of disease recurrence - a major problem in the management of PV-induced malignancies. Translation into immunotherapy of HPV16-induced cervical lesions is in progress.

Regulation of anti-tumor immune responses by sphingolipid metabolism in myeloid cells in melanoma

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Originally, metabolites are known to fuel bioenergetics and biosynthetic pathways, but in the past years it became clear that they often also regulate immune cell function, signal transduction and lineage commitment. Myeloid cells are very plastic and highly infiltrate tumors. In the tumor microenvironment, myeloid cells accumulate and often develop an immunosuppressive phenotype, weakening anti-tumor T cell responses and promoting tumor growth. Sphingolipids are a complex class of polar lipids with both signaling and structural functions. Consequently, sphingolipids are involved in the regulation of many (patho-) physiological processes such as cell proliferation, survival or death. The dysregulation of sphingolipid metabolism contributes to numerous pathologies, including inflammation and cancer. *De novo* biosynthesis of sphingolipids starts with the irreversible and rate-limiting condensation of L-Serine and palmitoyl-CoA to 3-ketosphinganine, catalyzed by the enzyme Serine palmitoyltransferase (SPT) with its enzymatically active subunit serine palmitoyltransferase long chain base subunit 2 (Sptlc2). This study investigates the role of Sptlc2 in myeloid cells on the regulation of the anti-tumor immune response in melanoma. In the *in vivo* mouse melanoma models, we observed that *Sptlc2*$^{flox/flox}$ Lyz2-cre mice developed significantly bigger B16 tumors. *Sptlc2* deficiency markedly affected the expression of various pro- and anti-inflammatory markers as MHCII and significantly reduced monocyte ($CD45^+CD11b^+Ly6G^-Ly6C^+F4/80^-$) and macrophage (Live $CD45^+CD11b^+Ly6G^-Ly6C^+F4/80^+$) numbers in the tumor. In Seahorse extracellular flux assays, both LPS, IFNγ-stimulated M1-like and IL4, IL13-stimulated M2-like bone marrow-derived macrophages (BMDM) showed reduced metabolic activity paired with impaired growth and proliferation upon *Sptlc2* deletion. *In vitro* supplementation of the Sptlc2 downstream sphingolipid Sphinganine rescued cell growth of Sptlc2-deficient BMDMs, whilst supplementation of the more complex sphingolipid Sphingomyelin did not. Moreover, confocal fluorescence microscopy revealed an impaired membrane localization and TLR4 co-localization of MyD88 in *Sptlc2*-deficient cells. In immunoblotting the strong increase in phosphorylated p65 at Ser536 in *Sptlc2*-deficient BMDMs confirmed a reduction of subsequent NFκB signaling. Overexpression of myristoylated, membrane-anchored MyD88 *in vitro* rescued the size of *Sptlc2*-deficient BMDMs. In conclusion, growth and proliferation deficits resulting from dysregulated pro-inflammatory signaling pathways of *Sptlc2*-deficient myeloid cells caused increased tumor growth, outlining the important role of intact sphingolipid metabolism on the anti-cancer function of myeloid cells and suggesting Sptlc2 as a potential drug target. The exact mechanisms how Sptlc2-derived sphingolipids regulate NFκB signaling remain to be studied.

The vicious cycle of melanoma-microglia crosstalk. Inter-melanoma variations in the brain-metastasis-promoting IL-6/JAK/STAT3 signaling pathway

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Previous studies from our lab demonstrated that the crosstalk between microglia and melanoma cells fuels melanoma brain metastasis progression. In the present study, an in-depth investigation of melanoma-microglia interactions elucidated a pro-metastatic molecular mechanism that drives a vicious melanoma-brain-metastasis cycle.

We employed RNA-Sequencing, HTG miRNA whole transcriptome assay and Reverse Phase Protein Arrays (RPPA) to analyze the impact of melanoma-microglia interactions on sustainability and progression of four different human brain-metastasizing melanoma cell lines.

We revealed a differential adaptability and response of these cellular interaction partners to cues delivered by the microenvironment. Microglia cells exposed to melanoma-derived IL-6 exhibited upregulated levels of STAT3 phosphorylation and SOCS3 expression, which in turn promoted melanoma cell viability and metastatic potential. IL-6/STAT3 pathway inhibitors diminished the pro-metastatic functions of microglia and reduced melanoma progression. SOCS3 overexpression in microglia cells evoked microglial support in melanoma brain metastasis by increasing melanoma cell migration and proliferation.

Although the different melanomas exhibited heterogeneity in their microglia-activating capacity as well as in their response to microglia-derived signals, we conclude that IL-6/STAT3/SOCS3 pathway activation in microglia is a major mechanism by which reciprocal melanoma-microglia signaling engineers the interacting microglia to reinforce the progression of melanoma brain metastasis. This mechanism may operate differently in different melanomas.

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Correlation of MSH2 exonic deletions and protein downregulation with breast cancer biomarkers and outcome among patients in Pakistan

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One of the underlying factors that contributes to the risk of developing breast cancer is the mismatch repair (MMR) pathway. In this study, the relationship between MSH2 exonic deletions, respective survival analysis, protein structure prediction, transcription profiling, and expression analysis with risk of breast cancer was investigated. Genotyping analysis of 230 breast cancer patients and 230 controls confirmed that there is an association between two MSH2 exonic deletions, exon 3 (OR:4.3, CI = 2.9–8.4) and 9 (OR:6.2, CI = 4.8–10.4), with risk of breast cancer. To see the relation between phenotypic genotypic, the investigators have performed MSH2 transcriptomic (p-value: 0.006) and protein expression analysis (OR:19.4, CI = 3–120) which confirm its downregulation in the biopsy samples of breast cancer and presenting potential role in the onset of carcinogenesis of breast. Furthermore, it is reported that MSH2 mutations can change expression profile of other biomarkers like ER, PR, CK–7, GATA–3, and E–cadherin associated with breast cancer. Later on, the effect of exonic deletions on secondary structure of protein has shown missing of beta and alpha helices in their protein products via in-silico analysis.

Nevertheless, loss of exon 3 resulted in the altered core protein structure leading to dysfunction protein, which may be the possible cause of development of breast cancer. There was no significant association between MSH2 exonic deletions with survival statistics reported the reason can be short follow-up time. Thus, our results at genetic, transcriptomic, and proteomic levels confirmed the downregulated MSH2, emphasizing its potential contribution in MMR mechanisms for breast tumorigenesis. In conclusion, deficiency of MSH2 can cause development and progression of breast cancer.

Cytokine-induced killer cell immunotherapy: expansion protocol optimization and its impact on cytotoxic activity

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Cancer immunotherapy has completely changed the approach to cancer treatment and has been able to improve its results by prolonging the survival of cancer patients with various tumors, improving their life quality and reducing side effects. Cytokine-induced killer (CIK) cell therapy is a type of cancer immunotherapy method that is being constantly developed and is based on manipulating autologous or allogeneic blood cells *in vitro* to kill cancer cells specifically. CIKs possess the properties of both T cells and natural killer (NK) cells and are characterized by a variety of subsets and $CD3^+CD56^+$ phenotype is the most prominent one. CIK cells can be manufactured by expanding lymphocytes using various cytokines such as IL-2, IL-15, IL-22, IFN-γ and anti-CD3 antibody and can be expanded to large, clinically relevant number of cells. In Lithuania CIK technology is relatively new and is still developing in a clinical setting. Importantly, the usage of different CIK manufacturing protocols offers the possibility to adjust their phenotype and modulate their cytotoxic properties.

In this work we attempted to create the optimal CIK cell expansion protocol using human peripheral blood lymphocytes from healthy donors, characterize the phenotype of those cells in more detail and perform a series of cytotoxicity assays. We investigated the cytotoxic effects of peripheral blood lymphocytes and CIKs not only on the K562 cell line, which is the golden standard for non-specific cytotoxicity testing, but we also used additional breast cancer cells lines that have different malignancy grade and chemotherapy resistance (doxorubicin (Dox): MCF-7 and MCF-7-Dox (luminal A type)) and MDA-MB-231 (triple-negative type). CIK cell expansion was performed using G-Rex membrane bioreactor setup, cell growth was identified by measuring glucose levels in growth medium and cell count was monitored using Trypan blue dye. Cytotoxic activity was measured with CCK8 and cell phenotype was established with flow cytometry using common CD markers (CD3, CD16, CD56) and additional markers specific to CIK activity (CD62L, CD45RA, CD95, CD107a, LAG3).

S100A9 and HMGB1 regulates the immunosuppression mediated by MDSC in melanoma

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Immunotherapeutic strategies in malignant melanoma have not yet reached their full potential mainly due to the resistance developed by a considerable number of patients. Myeloid-derived suppressor cells (MDSC), a heterogeneous population of myeloid cells, accumulate in the melanoma microenvironment. With the ability to inhibit anti-tumor T cell responses, MDSC are shown to promote immunosuppression, potentiate tumor progression and tumor cell resistance to the immunotherapy. TLR4 signaling was reported to be involved in the upregulation of PD-L1 expression and induction of immunosuppressive properties of human CD14+ monocytes. Damage associated molecular patterns (DAMPs) S100A8, A9, and HMGB1, which act as endogenous TLR4 ligands, drive MDSC activation and were shown to be markedly expressed in the tumor microenvironment (TME) of solid tumors. Nevertheless, the role of TLR4 signaling in the acquisition of MDSC immunosuppressive properties remains to be better defined. This study aims to evaluate the role of endogenous TLR4 ligands and TLR4 signaling on MDSC-mediated immune suppression in malignant melanoma.

MDSC were purified from peripheral blood of late-stage melanoma patients and in vitro generated from CD14+ monocytes from the peripheral blood of healthy volunteers. CD14+ monocytes were treated with recombinant (r) S100A9 and HMGB1 in the presence of GM-CSF for 72 h. The immunosuppressive capacity of MDSC and TLR4 ligand stimulated monocytes were assessed in the functional assays with T cells. In addition, TLR4 inhibitor (Resatorvid) and RAGE inhibitor (FPS-ZM1) were tested in T cell suppression assays. Markers and pathways involved in the MDSC immunosuppression were assessed by flow cytometry, Western blot, and gene expression profiling. The level of S100A8/9 and HMGB1 were measured in the plasma of melanoma patients by ELISA. TCGA data analysis was performed to evaluate the association between the expression of immunosuppressive markers and S100A9 and HMGB1 in the TME of melanoma.

Elevated plasma levels of S100A8/9 and HMGB1 were found to be correlated with the expression of immunosuppressive markers on peripheral blood MDSC in the melanoma patients. In addition, S100A8/9 and HMGB1 levels were markedly increased in the supernatant from in vitro generated MDSC. rS100A9 and rHMGB1 stimulated monocytes to acquire suppressive activity against T cells mediated by increased ROS and NO production as well as PD-L1 and IDO expression. The blockade of TLR4 signaling, and to a lesser extend RAGE signaling, led to substantial attenuation of the suppression of T cell proliferation. We suggest that both TLR4 and RAGE signaling pathways are involved in the acquisition of immunosuppressive properties of MDSC mediated by S100A9 and HMGB1.

NOTCH dependent cooperativity between myeloid lineages promotes Langerhans cell histiocytosis pathology

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Langerhans cell histiocytosis (LCH) is a potentially fatal neoplasm, characterized by the aberrant differentiation of mononuclear phagocytes, driven by mitogen-activated protein kinase pathway activation. LCH cells may trigger destructive pathology yet remain in precarious state, finely balanced between apoptosis and survival, supported by a unique inflammatory milieu. The interactions that maintain this state are not well-known and may offer targets for intervention. Here, we used single-cell RNA-seq and protein analysis to dissect LCH lesions, assessing LCH cell heterogeneity, and comparing LCH cells with normal MNPs within lesions. We found LCH-discriminatory signatures pointing to senescence and escape from tumor immune surveillance. We also uncovered two major lineages of LCH with DC2- and DC3/Monocyte-like phenotypes and validated them in multiple pathological tissue sites by high-content imaging. Receptor-ligand analyses and lineage tracing in vitro revealed Notch dependent cooperativity between DC2 and DC3/monocyte lineages, during expression of the pathognomonic LCH program. Our results present a convergent dual origin model of LCH with MAPK pathway activation occurring prior to fate commitment to DC2 and DC3/Monocyte lineages and Notch-dependent cooperativity between lineages driving the development of LCH cells.

Targeting a novel immune checkpoint molecule TAPBPL in antitumor immunotherapy

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Cancer progression is often accompanied by profound immune suppression that interferes with an effective anticancer response and cancer elimination. T cells are the major component in the immune system. Many cancers protect themselves from the immune system attack by producing immune checkpoint molecules to inhibit T cell functions. In the past several years, several new drugs that block immune checkpoint molecules PD-L1/PD-1 and CTLA-4 have been approved by the FDA for the treatment of various types of cancers. Although targeting PD-L1/PD-1 and CTLA-4 in the treatment of cancer has achieved unprecedented success, a complete and durable response is only seen in a fraction of treated patients. The results suggest that cancer cells and/or the cancer environment likely use additional checkpoint molecules to inhibit antitumor immune responses. The identification of additional immune checkpoint molecules is extremely important. We have used bioinformatics to identify a new immune checkpoint molecule, antigen processing binding protein like molecule (TAPBPL). TAPBPL shares sequence and structural similarities with the existing immune checkpoint molecules. TAPBPL protein is expressed on resting and activated T cells, B cells, monocytes, and dendritic cells, as well as in tumor tissues. The TAPBPL receptor is expressed on activated CD4 and CD8 T cells. Recombinant TAPBPL protein inhibits the proliferation, activation and Th1/Th17 cytokine production of T cells in vitro and in autoimmune disease animal models. In contrast, an anti-TAPBPL antibody neutralizes the inhibitory activity of TAPBPL on T cells. In vivo administration of the anti-TAPBPL antibody enhances antitumor immunity and inhibits tumor growth in animal models, which are related to increased percentages of tumor infiltrating CD4 and CD8 T cells but decreased percentage of regulatory T cells. Our results suggest that therapeutic intervention of the TAPBPL inhibitory pathway may represent a new strategy to modulate T cell-mediated immunity for the treatment of cancer, infections, autoimmune diseases, and transplant rejection.

The role of microRNAs derived from extracellular vesicles in the generation of myeloid-derived suppressor cells in malignant melanoma

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Due to the rapid progression and high metastasis capacity, malignant melanoma is the most invasive skin cancer, accounting for over 300,000 new cases and nearly 60,000 deaths each year. In advanced disease, conventional therapeutic approaches have limited efficacy. Despite the high prevalence of mutations in melanoma cells, activating an effective antitumor response is challenging. The tumor microenvironment, which is rich in immunosuppressive cells such as myeloid-derived suppressor cells (MDSC), is a serious impediment to this approach. MDSC can be generated by disturbed differentiation of immature myeloid cells (IMC) or by conversion of mature myeloid cells. Tumor-derived extracellular vesicles (EV) have the capability to activate immunosuppressive functions in mature myeloid cells. EV carry a variety of different molecular cargoes, including proteins, lipids, and RNA species such as microRNA (miR), which can be transferred to recipient cells or induce signaling from cell surface or intracellular receptors. Recently, a set of miR, found in melanoma patient-derived EV, has been identified to induce the conversion of normal human monocytes into MDSC. However, the mechanism by which the set of miR stimulates the generation of MDSC is still unknown. Therefore, we study how miR, present in melanoma-derived EV, can trigger the conversion of normal myeloid cells into MDSC. Treatment of murine IMC with miR-125a resulted in the upregulation of MDSC-related factors, including programmed death ligand 1 (PD L1), interleukin 6 (IL-6) and reactive oxygen species (ROS), indicating the acquisition of an immunosuppressive phenotype. In addition, gene expression analysis of IMC treated with miR-125a showed increased expression of genes belonging to Toll-like receptor (TLR), NF-κB and JAK-STAT signaling pathways, which play a role in the generation of MDSC. Since certain miR bind to endosomal TLR and can thereby contribute to the process of metastasis, the response of various TLR-deficient IMC to miR treatment was tested. Interestingly, the upregulation of PD L1 by miR-125a was independent of TLR signaling. However, NF-κB activation appeared to be crucial for miR-125a-mediated effects on IMC. Recent studies have shown that the cluster of miR-125a, miR-99b and let-7e is upregulated in human monocytes after prolonged TLR stimulation, leading to their conversion into immunosuppressive cells. It has been shown that the miR-125a/99b/let-7e cluster caused activation of MAPK/NF-κB/STAT3 signaling in human myeloid cells by targeting negative regulators of this pathway. Interestingly, the miR-125a/99b/let-7e cluster was overlapping with the set of miR found in melanoma patients. Therefore, we suggest that a direct transfer of the miR to normal myeloid cells by melanoma EVs in the tumor microenvironment could also induce similar effects, resulting in the generation of MDSC independent of TLR signaling.

Targeting tumor-associated macrophages in the immune-microenvironment of meningiomas

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Meningiomas represent the most common primary brain malignancies in adults with a subset of tumors that exhibit aggressive clinical behavior. Immunotherapy might present a new alternative treatment strategy but is highly dependent on the immunological composition of the tumor microenvironment. Our previous data have shown that tumor-associated macrophages (TAMs) make up the main immune cell population in meningiomas with a significant negative impact on patient outcome. In this study, we investigated whether TAMs from meningioma tissue could be reprogrammed to an immunologically active and tumoricidal phenotype. For this purpose, CD11b+ sorted macrophages derived from 40 patients were treated with small molecule inhibitors targeting the colony-stimulating factor-1 receptor (CSF-1R). In a first analysis, the direct treatment response of CD11b+ patient-derived macrophages was investigated by various techniques including flow cytometry and bulk RNA-sequencing of treated TAMs and further analysis of the macrophage-conditioned media after treatment. In addition, we studied the influence of CSF-1R-targeted macrophage treatment on the phenotype and functional activity of T cells to assess a potential indirect treatment response in the tumor microenvironment. Our data revealed that CSF-1R-targeted treatment of CD11b+ cells induced significant changes in the protein and gene expression of macrophage polarization markers towards a more immunologically active state and a significantly higher metabolic nitric oxide production as another sign of immunological activation. Subsequent analysis of indirect effects on T cells showed not only a significantly increased expression of the T cell activation marker CD69+, but also a significantly increased tumor cell killing by autologous T cells after macrophage-targeted treatment. Together these pilot data suggest both a direct and indirect CSF-1R-targeted macrophage treatment response in the local tumor microenvironment and give first promising results on the efficacy of macrophage-targeted immunotherapy in human meningiomas.

Androgen Receptor mediated sexual dimorphism in the progression of HNSCC

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For many cancers, including head and neck squamous cell carcinoma (HNSCC), women and men differ in terms of incidence, risk factors, prognosis, and treatment response. Although the issue is crucial, our knowledge on cellular and molecular principles underlying sex disparity in cancer is rather limited. The immune system's sex-specific function in cancer pathogenesis, progression, and therapy has been increasingly recognized. However, its implications in immunotherapy have been understudied. Therefore, comprehensive investigations are needed to understand the role of sex-specific immune responses in cancer and to develop effective immunotherapeutic strategies.

In this study, we generated pre-clinical mouse models using 4-Nitro Quinoline-1-Oxide (4NQO) - a tobacco surrogate and cancer cell lines derived thereof to address the mechanism of sexual dimorphism in HNSCC. From 4NQO-induced carcinogenesis, we found that male mice had a shorter survival rate and developed more severe disease than female mice. We also injected cell lines orthotopically to the lip of male and female mice of immuno-compromised (NSG) and immune-competent (WT) mice strains. We observed that male mice injected with the 4NQO cell line had faster tumor growth than female mice, but this difference was not observed in NSG mice of both sexes, indicating the importance of the immune system in mediating these sex-related differences.

HNSCC is often associated with chronic inflammation and has been linked to alteration in both sex hormone levels and the immune system, we used a novel approach of cross-species analysis and validation to explore the role of inflammatory mediators, sex hormones, and the tumor immune microenvironment as key modulators of sex-related differences in HNSCC by analyzing tumor tissue from pre-clinical models and patients. Our findings suggest that COX-2 (PTGS2, prostaglandin-endoperoxide synthase 2) staining patterns differ between male and female patients, with male patients showing predominantly positive staining in cancer cells and female patients showing predominantly positive staining in stromal cells in the tumor microenvironment. Also, the impact of inflammation on sex disparity was explored using IL-1b knock-out mice, which are known to exhibit lower inflammation and COX2 levels. 4NQO exposure revealed expected differences between sexes for IL-1b wildtype (+/+), but not knockout (-/-) mice strongly suggesting that sex-related differences in HNSCC pathogenesis are at least in part explained by the tumor immune microenvironment. Also, sex hormones have been reported to affect the fate of COX-2 expression during carcinogenesis and vice versa COX-2 modulates the expression of the androgen receptor (AR) and androgen inducible genes. Interestingly, blocking the AR using drugs such as Enzalutamide has been shown to inhibit cell proliferation in the 4NQO male cell lines. In addition to the in vitro study, we also analyzed the effect of enzalutamide on male mice with orthotopic tumor growth. Our results show that enzalutamide administration effectively inhibited tumor growth in the WT male mice and it did not show any effect in the NSG suggesting the role of AR in regulating the immune system. Hence, this study highlights the potential role of AR signaling in shaping the TIME in HNSCC and targeting AR signaling may reduce inflammation within the TIME and enhance the efficacy of immunotherapy in HNSCC.

T cell-derived Extracellular Vesicles (TEVs) are dynamic indicators of the immune response

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Immunotherapy with immune checkpoint inhibitors (ICI) targets inhibitory receptors on T cells, thereby reinvigorating anti-tumor immunity and inducing durable clinical responses in many cancer types. However, the number of responding patients remains globally modest. The limited therapeutic efficacy depends on different factors, including the generation of immune suppressive circuits hampering full-fledged anti-tumor immunity. Like any other cell, also T cells release extracellular vesicles (TEVs), which are detectable in peripheral blood. TEVs may not only represent a useful biomarker, but also contribute to the amplification or dampening of immune responses based on their phenotype and composition. An *in vitro* platform could be useful to identify the features of TEVs that are involved in promoting/inhibiting T cell activity.

We isolated TEVs from conditioned medium of 72 h TCR-triggered T cells (CD3/CD28 vs CD3 vs unstimulated) by differential centrifugations and analyzed the phenotype and molecular composition of cells and TEVs by Nanoparticle Tracking Analysis (NTA), flow cytometry, qPCR and transcriptomics. We also investigated plasma EV landscape to capture TEV nature in healthy donors by flow cytometry.

TCR triggering led to activation of T cells with concomitant increased production of TEVs, which closely resembled their originating cells, as shown by their immune RNA contents. Based on the stimuli, we could measure differences in concentration and RNA amount per TEV. Stimulated T cells also displayed higher EV marker (CD9, CD81, CD63) levels on their surface. TEV RNA analysis revealed differential microRNA content in samples derived from stimulated vs non-stimulated T cells. TEV transcriptomics evidenced a high similarity of profiles, despite derivation from different subjects, together with perturbations of IFN-related genes. Additionally, we could detect an upregulation of granzyme B (GZMB), IL2RA and other genes involved in different immune pathways (Th1, Th2) in TEVs from stimulated T cells, with respect to unstimulated. Plasma EV analysis revealed that in healthy donors only a small portion of EVs are T cell-derived EV.

Our results provide evidence about the feasibility of a TEV platform to capture the fine-tuning of TEV release, potentially depending on their functional fate. The TEV platform may enlighten new aspects of TEVs derived from T cells from cancer patients, who respond or not to ICI. Additionally, the TEV platform may be suitable to capture phenotype and molecular composition in other immunotherapy settings, such as CAR T cell therapies.

Modeling of dendritic cell immunotherapy for ovarian cancer

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Background. Dendritic cells (DCs) play a role in immunotherapy of ovarian cancer. According to the current range of ovarian cancer immunotherapy approaches, it is appropriate to improve dendritic cell vaccines (DCV) for better cancer management. We aimed to investigate allogenic DCV preparation with different ovarian cancer (OC) cell lines (A2780, SKOV3, COV362, OV7), their molecular profile, and their impact on dendritic cell maturation.

Methods. Flow cytometry analysis was used to determine the expression of CD44, CD73, CD105, CD274 stemness related markers in cancer cell lines. Gene expression was assessed by RT-PCR in OC cell lines. DCs were matured with prepared lysates of ovarian cancer cell lines and their mixture. Flow cytometry analysis was performed after DC maturation, surface markers such as CD11c, CD80, CD83, CD86, MHCII, and CCR7 were analyzed. Evaluation of gene expression in DCs was also performed by RT-PCR. Pearson correlation analysis was used to investigate the relationship of the ovarian cancer cell line molecular profiles and DC maturation level.

Results. Studied OC cell lines have different molecular profiles. Individual OC cell lines and their mixture have different effects on the maturity of DCs. The highest expression of genes associated with immunogenicity and the lowest with tolerogenicity – were achieved using a mixture of all OC cell line lysates for the maturation of DCs. The OV7 cell line with the mesenchymal and stem-like phenotype is able to induce the highest expression of maturation markers on DCs. Genes encoding the major transcription factor for epithelial-mesenchymal transformation SNAIL1 and the multidrug resistance active transport carrier ABCG2 are strongly correlated with essential DC maturation markers CD80 and MHCII and CD83/CD86 and STAT1 transcripts ($p < 0.05$).

Conclusion. In early phase clinical trials where it is not always possible to use the patient's autologous tumor material could be used the mixture of OC cell lines lysates for allogenic DCV preparation. Single-cell transcriptional profiling could be further investigated to improve allogenic DCV immunotherapy of ovarian cancer.

The Involvement of Myeloid-Derived Suppressor Cells in Inflammatory Bowel Disease

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Inflammatory bowel disease (IBD) represents a chronic inflammatory condition that when uncontrolled, predisposes the host to colitis-associated colorectal cancer (CAC). Both IBD and CAC are characterized by dysregulated immune systems and changes in the intestinal microbiota composition. The dysbiotic bacteria invade the intestinal tissue and are found to interact with diverse immune cells of which one key population is represented by myeloid-derived suppressor cells (MDSCs). Bone marrow precursors of MDSCs are a heterogeneous population of immature cells, that under normal conditions give rise to immune cells of the myeloid lineage. However, under pathologies associated with chronic inflammation as in IBD, these cells become polarized MDSCs that display highly suppressive activities. MDSCs accumulate in the site of inflammation and periphery and impose their suppressive effect on both the innate and adaptive immune systems by different routes. Thus, both the changing microbiota and MDSCs contribute to the progression of IBD toward CAC through the induction of persistent immunosuppression and a proinflammatory environment in the colon. MDSC-mediated suppression is linked to the secretion of proinflammatory cytokines and nitric oxide, the arrest of T cell proliferation, and downregulated levels of CD247, which is a key signaling molecule expressed by T and NK cells. Since MDSCs are a major cell population that dysregulates the immune system, they are major obstacles to the success of various immune-based therapies. Therefore, different anti-MDSC therapeutic strategies were tested in recent years towards recuperating the host's health conditions and disease regression. Thus, finding novel pathways that mediate MDSC-induced immunosuppression, is necessary to allow the development of anti-MDSC therapeutic modalities. Herein, we will present a strategy that alleviates intestinal inflammation by manipulating MDSC-induced immunosuppression, and more specifically, will discuss the plasticity of MDSCs during IBD progression and the use of unique metabolites as MDSCs' inhibitors.

Modulation of NK cell cytotoxicity by impairing cystatin F activation

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Natural Killer (NK) cells are innate immune cells that play a crucial role in cancer immune surveillance. The granzyme perforin pathway is a major mechanism by which NK cells kill target cancer stem cells. It is regulated by proteolytic activation of granzymes and perforin by cathepsins C, H, and L. However, NK cells are susceptible to the immunosuppressive factors present in the tumor microenvironment, which can impair their cytotoxic function. One of these factors is cystatin F, a member of the cystatin family of protease inhibitors that can inhibit cathepsins C, H, and L and therefore negatively impacts NK cytotoxicity. We have identified cystatin F as an important immunosuppressor in glioblastoma tissue. Although cystatin F is normally expressed in immune cells, its levels were found to be increased in glioblastoma tissue, where also tumor cells expressed cystatin F. Cystatin F function is regulated by expression levels, N-glycosylation, and proteolytic activation. In the lysosomes, cystatin F is activated from inactive dimeric form to active monomer by cathepsin V, which cleaves 15 N-terminal amino acids from cystatin F. The main objective of this study was to evaluate NK cell function in glioblastoma patients and to improve the cytotoxicity of NK cells towards glioblastoma stem cells by preventing cystatin F activation.

The cytotoxic function of healthy donor NK cells is superior to patient-derived NK cells, as evaluated by calcein release cytotoxicity assay. However, healthy NK cells are still susceptible to the effects of cystatin F. Using flow cytometry cytotoxicity assay, ELISA and enzyme kinetics, we have shown that recombinant cystatin F caused decreased cytotoxicity, increased IFN-γ secretion, and decreased activity of cathepsin C and granzyme B. To mitigate the effects of cystatin F on NK cell cytotoxicity we have developed a new small molecular inhibitor of cathepsin V, the activating peptidase of cystatin F. We used molecular docking of small molecular compounds from commercial libraries with cathepsin V and evaluated selected compounds by enzyme kinetics for the enzyme inhibition, selectivity, and reversibility of binding. The effect of the most potent, selective and reversible-acting cathepsin V inhibitor on cystatin F activation was tested by western blot. Cathepsin V inhibition decreased the conversion of cystatin F from dimer to active monomer form in primary NK cells. Furthermore, primary NK cells treated with cathepsin V inhibitor had increased cytotoxicity against glioblastoma stem cells compared to untreated NK cells.

To conclude, selective inhibition of cathepsin V prevents the monomerization and activation of cystatin F. By targeting cystatin F activating peptidase, we can reduce the detrimental effects of cystatin F on NK cells and increase their cytotoxic potential.

Understanding the tumor microenvironment: mapping the spatial distribution of immune cells in cervical and HPV+ head and neck squamous cell cancer

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Background: The tumor microenvironment (TME) consists of a heterogenous population of immune cells, networks of fibroblasts, blood vessels, lymphatics, signaling molecules and the cancer itself. The composition of the TME differs between patients but also between different kinds of tumors. Understanding the composition of the TME is crucial for tailoring of immuno-therapies that may increase chances for a successful response in patients. Hence, filling knowledge gaps regarding the spatial distribution of leukocytes in cancers is imperative in our quest to beat cancer.

Methods: Immunostainings were conducted in HPV+ head and neck squamous cell cancer (HNSCC) (*n*=62) and cervical cancer (CESC) (*n*=71) samples to compare the immune profile between the two indications. Additionally, the CIBERSORT-ABS tool was used in patients selected from the TCGA database. To detect chemokine expression in tumor cells, we purified EpCAM+CD45-CD90- expressing cells from native carcinoma samples and performed RNA-sequencing.

Results: We found that the distribution of immune cells in the TME markedly differs between HPV+ HNSCC and CESC with substantially higher levels of immune cells in HPV+ HNSCC tumor epithelia, except for myeloid-derived suppressor cells, which were more abundant in CESC. We also found significantly higher levels of tertiary lymphoid structures in the TME of CESC samples. Furthermore, classification of the tumors as cold, hot, and excluded was done based on cut-off values of CD8+ T cells showing that 92.3% of HPV+ HNSCC samples rank among hot tumors whereas only 40.3% of CESC samples can be considered as such. On the contrary, the proportions of samples with excluded TME were 6.2% and 28.4% % in HPV+ HNSCC and CESC, respectively. Similarly, the proportions of immunologically cold samples were 1.5% and 31.3% in HPV+ HNSCC and CESC, respectively. Thus, hot TME markedly prevails in HPV+ HNSCC, whereas the distribution of hot, excluded, and cold TME can be considered equal in CESC. Lastly, based on the RNA-sequencing, we found increased levels of chemokines associated with neutrophiles and myeloid-derived suppressor cell trafficking, namely CXCL1 and CXCL5 in CESC derived tumor cells and significantly higher levels of CXCL9 and CXCL10 in HPV+ HNSCC tumor cells.

Conclusion: The spatial distribution of immune cells differed between HPV+ HNSCC and CESC with in general higher levels found in HPV+HNSCC except for myeloid-derived-suppressor cells. Furthermore, the chemokine profile from tumor cells differed between the two indications showing an increased affinity towards neutrophile and myeloid-derived suppressor cell trafficking in CESC samples. This understanding of TME variability may give us clues on how the patients might differ in their needs regarding targeted immuno-therapy.

The Importance of Immune Suppressive Microenvironment in the Development of Glioblastoma

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Glioblastoma multiforme (GBM) is the most frequently occurring primary brain tumor, and patients' survival is 12-15 months following diagnosis, despite all invented therapies and treatment strategies. GBM combines a lack of immunogenicity and a highly immunosuppressive tumor microenvironment (TME). Tumor cells, endothelial cells, immune cells, and a variety of cytokines secreted by the cells regulate immune effects in the TME.

The multicellular spheroid (MCS) model system allows us to investigate molecular tumordeveloping mechanisms similar to in vivo micro-environmental conditions, including hypoxia, cellcell contact, oxygen, and nutrient gradient. This study aimed to evaluate the changes in genome-wide gene and miRNA expression in human glioblastoma U87 cells in the 3D multicellular spheroid culture model, which accurately represents in vivo tumor niche. Microarray analysis revealed a total of 926 significantly differentially expressed genes. Next-generation short non-coding RNA sequencing identified 62 miRNAs with a significant expression change. Data from these genome-wide expression analyses were validated using RT-qPCR.

Bioinformatical enrichment analysis of microarray data identified 25 significantly enriched KEGG functional gene groups, which were divided into four major categories: immune response-related, cell adhesion, metabolism, and other functional groups. Metabolic mapping analysis showed that the main expression profile arises from upregulated genes involved in the autoimmune response, angiogenesis, and cell adhesion mechanisms. Moreover, investigation of KEGG pathway maps led to the hypothesis that transcription in glioblastoma cells is hypoxia-induced and regulated through a nuclear hormone receptor RORA, which is activated by cholesterol-derived ligands via activation of the steroid biosynthesis pathway. Understanding how immune response mechanisms interact with one another, as well as how it affects the tumor-microenvironment, will provide experimental evidence for the comprehensive diagnosis and treatment of gliomas.

Relationship between the senescence and exhaustion status of donor lymphocytes with the cytotoxic activity of CIKs

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Cytokine-induced killer cells (CIKs, CD3+CD16/56+) remain a promising direction of antitumor adoptive immunotherapy. Challenges in obtaining CIKs that are highly active against tumor cells and resistant to the immunosuppressive tumor environment are still relevant. Therefore, different sources of lymphocytes (peripheral blood or umbilical cord blood) and conditions of their cultivation with the optimal cytokine cocktail are being actively investigated. Determination of the senescence and exhaustion status of donor peripheral blood lymphocytes is crucial to a successful production of CIKs. There is sufficient data accumulated today on the mechanisms of lymphocyte senescence and depletion in various physiological (aging) and pathological (tumor disease) conditions to indicate that cancer patient-derived lymphocytes can be problematic in response to activation and proliferation stimuli. Moreover, premature aging of blood lymphocytes under the influence of such negative factors as chemotherapy, radiation therapy and stress is ever so important in oncological patients, making pre-treated autologous blood donors a potentially suboptimal choice. We selected a number of indicators that characterize the status of aging and exhaustion of lymphocytes and characterized donor lymphocytes before the start of their cultivation in the G-Rex membrane bioreactor. We also conducted an analysis of the relationship between the status of senescence and exhaustion of lymphocytes with the cytotoxic activity of CIKs obtained according to different protocols.

The functional effects of PMN-MDSC on PD-1^{+} and PD-1^{-} effector memory CD4^{+} T cells under PD-1 immune checkpoint blockade

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In the case of chronic inflammation such as cancer, prolonged inflammatory signals and over expression of growth factors stimulate the bone marrow to meet the increased demand for myeloid cells. This results in an emergency myelopoiesis in which the immature myeloid cells fail to differentiate into mature cells before their egress from the bone marrow. In recent years, it has become evident that the abnormal accumulation and function of the immature myeloid cells are important facets of the cancer. MDSC are composed of immature myeloid cells at different stages of myelopoiesis and are identified with certain myeloid lineage markers. As common properties of MDSC, they have low-density (<1.077 g/mL) and display HLA-DR$^{low/-}$ immunophenotype. MDSC are further categorized into two major groups as monocytic MDSC (M-MDSC) and granulocytic/polymorphonuclear MDSCs (PMN-MDSC).

This study aims to analyze the suppressive effects of PMN-MDSC on PD-1^{+} and PD-1^{-} effector memory CD4^{+} T cells in the presence of PD-1 blockade. Peripheral blood samples of 8 colorectal cancer, 2 melanoma patients, and 6 healthy donors were collected. Peripheral blood samples were layered over 1.077 g/mL Histopaque solution and PBMC were collected. Cells were labelled with anti-human -CD45, -CD11b, -CD33, -CD14, -CD66b, -PD-L1, -HLA-DR, -CD3, -CD4, -CD8, -PD-1, -CCR7, -CD45RA, and -CD45RO monoclonal antibodies. The frequency of total and PD-L1-expressing PMN-MDSCs was increased in the patients. Amongst the central memory (T_{CM}), effector memory (T_{EM}), terminally differentiated effector memory (T_{EMRA}), and naïve (T_{Naive}) CD4^{+} T cell populations, T_{EM} was the most prominent subtype in which PD-1 expression (median 35%, min 4.4% - max 54.4%) was detected. Next, CD4^{+}CD45RO^{+}CCR7^{-}PD-1^{-} and CD4^{+}CD45RO^{+}CCR7^{-}PD-1^{+} T cells were purified by FACS. CD11b^{+}CD33^{dim}HLA-DR$^{-/low}$CD66b^{+} PMN-MDSC were purified with MACS followed by FACS. CFSE-labelled autologous T cells were stimulated with CD3/CD28 beads and co-cultured with PMN-MDSCs in the presence or the absence of anti-PD-1 mAb. After 72 hours of incubation, PMN-MDSC suppressed proliferation of both PD-1^{+} and PD-1^{-} T_{EM} CD4^{+} T cells. Although PD-1^{+} T_{EM} CD4^{+} T cells were more prone to immune suppression by PMN-MDSC, PD-1 blockade recovered T cell proliferation in both groups to certain extent.

VHL inactivation confers decreased infiltration and killing capacity of NK cells in clear cell renal cell carcinoma

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Von Hippel-Lindau (*VHL*) inactivation in clear cell renal cell carcinoma (ccRCC) results in stabilization of HIF, increased HIF transcriptional activity and up-regulation of HIF target genes such as *VEGF* and *PDGF*. With ccRCC being an immunogenic tumor, it is important to understand how the loss of VHL affects the anti-tumor immunity. Here we sought to investigate the role of ccRCC *VHL* status and its impact on NK cell function. Upon restoration of *VHL* (786-O-pVHL), ccRCC spheroids were less hypoxic and displayed a less invasive phenotype compared with *VHL* inactivated ccRCC tumors (786-O). Mass spectrometry analysis revealed proteomic changes upon *VHL* restoration including the downregulation of ccRCC progression and immunosuppressive pathways. When added to ccRCC spheroids, NK cells showed a 2.5-fold increased infiltration into 786-O-pVHL spheroids compared with 786-O spheroids as measured by flow cytometry (p = 0.0012). In addition, 786-O-pVHL infiltrating NK cells displayed reduced expression of HIF1α (1.89-fold, p = 0.0051) and PD-1 (2.6-fold, p = 0.0018). Furthermore, NK cells displayed higher levels of IFN-gamma (1.8-fold, p = 0.0024) and CD107a (2.2-fold, p = 0.0380), and an increased ability to induce apoptosis of 786-O-pVHL compared with 786-O spheroids. The ability of NK cells to induce apoptosis of 786-O-pVHL was also confirmed in a kidney tumor organoid model. Altogether, we show that *VHL* inactivation in ccRCC reduces infiltration and the killing ability of NK cells, and NK cells in VHL restored spheroids display a more activated and mature phenotype accompanied with reduced expression of immune checkpoint receptors. Thus, *VHL* restoration may represent an attractive approach to improve NK cell infiltration and function in ccRCC.

Transcriptional and Functional Analysis of CD1c$^+$ Human Dendritic Cells Identifies a CD163$^+$ Subset Priming CD8$^+$CD103$^+$ T Cells

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Dendritic cells (DCs) act as sentinels of the immune system as they possess the superior ability to prime and activate T cells. In humans, the diversity, ontogeny and functional capabilities of DC subsets are not fully understood. Historically, DCs have been divided in plasmacytoid DCs and classical DCs (cDCs), where cDCs are further separated in cDC1 and cDC2. However, recent findings have shown further heterogeneity within the DC population with the addition of DC3. We have reinforced these finding by identifying circulating CD88$^-$CD1c$^+$CD14$^{+/-}$CD163$^+$ DC3 as an immediate precursor of inflammatory CD88$^-$CD14$^+$CD1c$^+$ CD163$^+$FcεRI$^+$ DC. *In vitro*, in contrast to cDC1 and cDC2, growth factor GM-CSF and not FLT3L promotes the expansion of DC3. DC3 also develop via a specific hematopoietic pathway activated by GM-CSF, independent from the cDC-restricted (CDP) and monocyte-restricted progenitors (cMoP). Furthermore, we show that DC3-commited progenitor exists in the CLEC12A$^+$ fraction of granulocyte, monocyte and DC progenitor (GMDP). *In vivo*, DC3 can infiltrate humanized mouse B16 lung tumours expressing GM-CSF and human luminal breast cancer primary tumours. Furthermore, DC3 show a superior ability to induce the proliferation of CD8$^+$CD103$^+$ T cells, termed tissue-resident memory T cells (T_{RM}), and the DC3 infiltration correlates positively with T_{RM} in luminal breast cancer patient samples. Collectively, this data defines DC3 as plastic but specialised DC subset with a distinct origin, developmental requirements and specific functional capabilities.

B7-H3 specific CAR-CIK lymphocytes effectively kill lung cancer cells including chemoresistant cancer stem cells

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Purpose of this study is to explore a novel CAR-based cellular immunotherapy against non-small cell lung cancer (LC), including the emerging subset of cancer stem cells (LCSCs) considered responsible for chemoresistance and disease relapses. The strategy is focused on Cytokine-Induced Killer lymphocytes (CIK) redirected against B7 homolog 3 protein (B7-H3), associated with immunosuppressive functions and stemness. CIK are *ex vivo* expanded T-NK lymphocytes endowed with intrinsic HLA-independent anti-tumor activity (NKG2D mediated). The underlying rationale is that CAR engineering of CIK would then result in a dual tumor cell killing potential.

Experimental procedures. The experimental platform is based on cancer patient derived CAR.CIK and multiple LC cell lines. CAR.CIK were generated by transduction of PBMC with a retroviral vector encoding for a 2nd generation anti-B7H3 CAR with CD28 co-stimulation. To visualize and track the fate of putative LCSCs, LC cells were engineered with a lentiviral CSC-detector wherein the promoter of stem-gene Oct4 controls the expression of eGFP, previously validated in our lab (Gammaitoni et al, Clin Cancer Res 2017).

Results. CAR.CIK were efficiently generated by cancer patients (n=3). Mean expression of anti-B7H3 CAR was 40±7%, CIK subsets (CD3+CD56+: 45±11%, CD3+CD8+: 59±14%, NKG2D: 84±3%) were comparable to paired non-transduced CIK (NTD.CIK). The CAR-target, B7-H3, was intensely expressed by LC cells (87±5%; adenocarcinoma n=8, squamous cell carcinoma n=2,) while NKG2D ligands showed a variable expression with MIC A/B = 5% (range 0-98) and ULBPs 2/5/6 = 87% (range 18-99). We could visualize a subset of GFP+ LCSCs (9±2%, n=3), that were confirmed to be relatively chemoresistant, as their rate increased (1.5 fold) after treatment in vitro with cisplatin. CAR.CIK efficiently killed LC *in vitro* and their antitumor activity was significantly superior as compared with control NTD-CIK, especially at low effector/target (E/T) ratios (95% vs 80% E/T 10:1; 61% vs 23% E/T 1:2, $p<0.005$, n=8). Anti-B7-H3 CAR.CIK effectively eliminated also the subset of chemoresistant GFP+LCSCs (100% E/T=5:1 and 66% E/T=1:2).

Conclusions. Anti-B7-H3 CAR.CIK effectively kill NSCLC cells *in vitro*, including the eradication of chemoresistant LCSCs. CIK are a promising platform for CAR engineering approaches and our findings provide rationale to explore this approach in clinical studies within the setting of NSCLC resistant to conventional treatments.

Ovarian cancer cell 3D spheroids as a platform for immune excluded tumor modelling

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Epithelial ovarian cancer (EOC) is the leading cause of death among gynaecological malignancies. The high level of EOC heterogeneity contributes significantly to the lack of successful treatments [1]. To improve our understanding of epithelial ovarian tumour biology and behavior, it is important to adopt a relevant culture system. Usually, the majority of in vitro experiments are performed in two-dimensional (2D) cancer cell line monolayer cultures. Even though the 2D model is commonly used, it does not fully reflect the tumour complexity, therefore, more sophisticated models should be adopted. Cancer cell microstructures formed as three-dimensional (3D) spheroids closely represent characteristics of in vivo tumours, such as cell-cell interactions, hypoxia and pH rate, exposure to nutrients and metabolites, and gene expression profiles [2]. On the other hand, 3D models are usually composed of only one cell type and, more often than not, an immortalized cell line. To be more physiologically relevant, the model should also address more complex cellular interactions within the tumor environment.

In our study, we aimed to adopt and optimize the 3D culture conditions of four molecularly and morphologically different EOC cell lines: A2780, SKOV3, COV362, OV7 and their co-cultures with ovarian tumor-derived fibroblasts. Next, using quantitive real-time PCR (qPCR) we compared the gene expression profiles between 3D EOC cell models and EOC cell co-culture with fibroblasts models and widely used 2D cell culture models by analyzing the expression of genes related to stemness properties, epithelial-mesenchymal transformation and interaction with the immune system.

3D spheroids were measured and observed in different growth conditions and at a different starting point seeding densities. We noticed that the A2780 cell line did not form spheroids unless the fibroblasts were also present in the co-culture. After optimization and determination of the best cell count and conditions for 3D culture, gene expression profile evaluation was performed. We found that spheroid formation process induced distinct transcriptional changes in different models. Notably, VEGF was increased in SKOV3 and COV362 3D models in comparison with respective 2D cell lines. More, POU5FA, SOX2 expression was increased in SKOV3, COV362, OV7 3D models. Next, we compared the gene expression in respective 3D cultures and 3D co-cultures with fibroblasts, where we also found substantial differences. Our findings will be crucial in the future creating and developing the best models to reflect immune excluded tumors in vitro.

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Atezolizumab as potential inhaled immunotherapy agent and lectin influence on the efficacy of Atezolizumab on prostate cancer cells DU-145

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Protein-based anticancer drugs such as immune checkpoint inhibitors (ICI), including atezolizumab, that targets PD-L1, expressed on tumor cells, are expected to be the most, appealing candidates for use in inhalation therapy, because of safe profile in contact with tissues and low susceptibility to nebulization process using vibrating mesh. Despite increasing use of atezolizumab, it is also well known that PD-L1 undergoes posttranslational modifications, such as N-glycosylation, which is important regulatory mechanism in immunosuppression modulation in cancer patients, therefore targeting PD-L1 glycosylation could be used to improve anticancer immunotherapy. Lectins, which are carbohydrate binding, non-immune origin proteins exhibiting potential immunomodulatory and antitumor properties, may potentiate the effect of atezolizumab.

Overall, this study aimed to determine whether different forms of immunomodulatory drug atezolizumab affects its effectiveness in blocking membrane PD-L1 in human prostate cancer cell line DU-145, and whether lectin *Bacillus subtilis* has any influence on the ability of atezolizumab to block PD-L1 ligand on DU-145 cells.
In the atezolizumab binding experiments, cells were plated, allowed to adhere for 24 h, and then treated with atezolizumab in different forms: native and nebulized. Each form was tested in DU-145 cell binding assays at different concentrations and incubated at 37°C. For the lectin in cooperation with atezolizumab binding experiments, cells were processed as described previously at two different concentrations of lectin. All samples were analyzed by flow cytometry method.

The findings suggest that inhibition of anti-CD274 staining intensity of DU-145 cells, expressing PD-L1 ligand on their surface, depends on the concentration of anti PD-L1 drug atezolizumab. The nebulized form of atezolizumab retains cellular binding to PD-L1 and is equally effective as its native form therefore could be potentially used for inhalation therapy. *Bacillus subtilis* lectin does not bind the membrane PD-L1 in DU-145 cells and does not influence the ability of atezolizumab to block PD-L1 ligand, when incubated together. However, the study found that exposure to lectin first, and then subsequently to atezolizumab, slightly reduces the ability of atezolizumab to block the membrane PD-L1. Further investigations are needed to obtain more detailed information about the impact of lectin on the effectiveness of atezolizumab.

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