Open flow microperfusion (OFM) enables animal experiments with a high data output that leads to reduced animal numbers

Thomas Birngruber

Open flow microperfusion (OFM) is a probe-based method that is used to evaluate the pharmacokinetics and pharmacodynamics of drugs directly in skin, adipose and brain tissue. The use of the OFM probes allows small amounts of interstitial fluid (ISF) to be collected from target tissues for analysis. OFM is particularly suitable for the analysis of large, lipophilic substances. OFM experiments deliver time resolved compound profiles and provides therefore a series of data points from one animal that is unparalleled.
In the field of dermatology the OFM technology provides a higher data quality compared to standard methods like skin biopsy that provide a benefit in the power calculation and therefore leads to a reduction in required animals.
This high data quality enables a data modelling and extrapolation approach based on bootstrap, PBKD and T-cat algorithms that allow to further reduce or even replace animal experiments.
The talk will illustrate this topic with practical examples and published data.

Mechanical Stimulation as a tool in osteoarthritis research

Birgit Lohberger, Department of Orthopedics and Trauma, Medical University Graz, Graz, Austria
Bibiane Steinecker-Frohnwieser, Ludwig Boltzmann Institute for Arthritis and Rehabilitation, Saalfelden, Austria

Osteoarthritis (OA) is a chronic joint disease that damages the joint cartilage and surrounding tissue. Typical symptoms are pain, joint stiffness and loss of function. Physical activity not only contributes to well-being and increased quality of life, but also plays an essential role in the prevention of diseases. Many studies on the mechanical stimulation of cartilage cells and tissues are concerned with the question of how mechanical signals influence gene expression.
In general, we distinguish between three different types of mechanical stress: tension, compression and shear stress. One device for applying tension is the Flexcell® System (FX5K), which allows the cells to be exposed to cyclic or static tensile stresses. Special BioFlex® culture plates with a flexible membrane are used and different forms of mechanical stimulation (sinus, triangle, square or a typical heart waveform) can be applied (frequency and amplitude are variable). The BioFlex® plates are available with different coatings (Collagen Type I or Type IV, ProNectin, Laminin) in two-dimensional cell cultures or special plates for tri-dimensional (3D) applications. The choice of the appropriate plates depends on the cell type and the respective research question. Additional equipment for the FX5K® tension system allows the application of shear-stress, co-cultures (Transwell® holder), uni-axial stimulation (Uni-Flex® culture plates), and 3D cultures (Tissue Train®).
Depending on the question, the choice of stimulation protocol is of great importance. In the case of OA, strong mechanical stress on the OA chondrocytes causes a decrease in the expression of anabolic genes and increased cartilage degeneration. A moderat mechanical stimulation and a physiological movement pattern increases the expression of collagen (Col2A), aggregan and extracellular matrix components significantly and effects increased cartilage regeneration.

Increasing the knowledge gain per animal used

Bernhard Voelkl

According to the prevailing view, the reproducibility crisis in pre-clinical animal research is caused by a lack of scientific rigor, low statistical power, and publication bias. However, ignorance of biological variation that we will encounter whenever conducting an experiment with living animals might be a major reason for irreproducibility of research findings. Biological variation is the sum of genetic variation, environmentally induced variation, and gene-by-environment interactions. Ignoring the implications of biological variation is likely to lead to spurious results that are idiosyncratic to the specific standardized laboratory conditions, thereby causing poor reproducibility. Irreproducible results generate the need for follow-up studies requiring further animals, or might—in the worst case—lead researchers into scientific dead ends, wasting even more animals for studies that cannot provide any benefits in terms of knowledge gains. Implementing experimental designs that embrace biological variation can increase the external validity and the reproducibility of animal experiments and can, therefore, increase the knowledge gain accrued per animal used.

aCTM MUG CCArly 1 – a new autologous 3D cholangiocarcinoma co-culture tumor model

Silke Schrom

Cholangiocarcinoma (CCA) belongs to the group of rare cancers with an incidence of 0.3 – 6 in 100 000 people yearly. This type of cancer originates is the biliary tract. Patients are often diagnosed in advanced stages due to the lack of specific symptoms. CCA are resistance to chemo- and radiotherapy and the only curative treatment option is surgery at early stages. Therefore CCA contributes to 2% of all cancer-related deaths with a 5-year survival rate of 7 – 20%.
CCA are highly desmoplastic, often composed of a higher percentage of cancer-associated fibroblasts (CAF). These CAFs contribute to tumor progression, angiogenesis, invasion and metastasis. To this day, not many CCA in vitro models exist, taking the pivotal role of CAFs into account.
We present a new established, well-characterized CCA model – MUG CCArly 1 and it's autologous immortalized CAFs. In our 3D co-culture model we demonstrate the pivotal role of CAFs in angiogenesis and compare anti-cancerous peptides in 2D- and 3D cultures. Our 3D model even presented markers, indicating an epithelial-to-mesenchymal (EMT) character.
This new 3D aCTM co-culture model can be of use for cross-talk studies and the testing of new therapy approaches in CCA research.

Unrelieved pain and what it does to your experiments

Akos Szakmary

Sometimes researchers are reluctant to provide analgesia because it has side effects and they argue it might affect their data. This has not only ethical problems but also scientific ones.
The EU-Directive states that no one may cause an animal pain, suffering or harm without good reason – and that pain, suffering, or harm may only be inflicted on the animals when unavoidable to attain the purpose of the experiment. In particular, they must not be inflicted in order to save work, time or costs.
So why do researchers planning painful procedures often resist analgesia on grounds it might confound results or disallow comparison with older data?
While they do NOT take into consideration that unrelieved pain might alter their results, or confound measured effects, cause unnecessary suffering, force premature termination of their experiment or that unrelieved pain is not really an option in a clinical setting and their results might lose relevance?
This talk gives a short overview of physiological systems potentially affected by pain.
Pain has pathophysiological effects that can bias experimental results in a non-quantifiable manner. This implies, of course, also insufficient or wrongly timed pain management. This bias can be reduced by adequately administering analgesics.

The Establishment of EU3Rnet: A Network of European 3Rs centers and societies

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The aim of EU3Rnet is to bring together European 3R centres and societies to share best practices, improve communication, support information exchange and prepare the ground for joint initiatives.
After a first meeting of representatives of 3R centres and societies at the EUSAAT conference in Linz (Austria) in September 2018, follow-up meetings were held in March 2019 after invitation of the Free University of Berlin and at the FELASA conference in Prague in June 2019.
In the discussions it became clear that the diversity of the members could be the strength of the network as they cover many different topics and have experts in refining, reducing and replacing animal testing.
Important common goals were identified, in addition to joint COST proposals, a CONSENSUS STATEMENT was published in 2020. Publications on the rise of the 3Rs centres, current tasks and future goals are currently in progress.
The network is a fully independent, open and free community, highly dependent on the initiatives of its protagonists and personal efforts. It is based on a bottom-up approach and any 3R centre or society is welcome to join.

Reference: Neuhaus W “Consensus Statement from the European Network of 3R Centres (EU3Rnet)”, ALTEX 2020, doi: 10.14573/altex.2010061, e-mail: winfried.neuhaus@ait.ac.at

The mouse model in SARS-CoV-2 research Part I: The Betacoronavirus

Philipp Hohensinner

The current SARS-CoV-2 betacoronavirus is causing a massive pandemic. However, SARS-CoV-2 is not the first betacoronavirus to be infectious for humans. However, beta-coronaviruses are not novel and already several human specific beta coronaviruses were reported. Also in the mouse, the mouse coronavirus or better known as mouse hepatitis virus is a beta coronavirus. The mouse specific strain is already known since the 1950ies and has been extensively studied. In this talk we will discuss similarities and differences between mouse and human virus and caused symptoms. We will further define to what extent the mouse virus can be used as a model system to allow for drug development and testing.

The mouse model in SARS-CoV-2 research Part II: **Innate immune training with bacterial extracts enhances lung macrophage recruitment to protect from betacoronavirus infection**

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Background:
We analyzed if a prophylactic immune cell training using the bacterial extract Broncho-Vaxom OM-85 (BV) is beneficial during a coronavirus infection and could thereby assist in controlling this pandemic.

Methods:
We trained the immune system with BV before a coronavirus infection using the murine coronavirus mCoV A59 in a mouse model.

Results:
Training of the immune system led to reduced viral load and reduced lung tissue damage. RNA sequencing showed that lung tissue of treated mice resembled an intermediate state between infected and healthy tissue. On a cellular level, treatment with BV increasing numbers of interstitial lung macrophages, without affecting overall inflammation or endothelial cell activation. We were able to phenocopy the effect of BV by transplanting naïve lung macrophages into recipient mice thereby enhancing the local cellular amount before infection, and inhibition of interferon type I signaling negated the positive effect of BV.

Conclusion:
BV oral intake selectively enhanced the innate immune system in the lung allowing for a faster immune reaction towards a coronavirus infection. We conclude that this training of the innate immune system by the bacterial extract BV boosts interferon type I response and is thereby beneficial during a coronavirus infection.

The mouse model in SARS-CoV-2 research Part III: Host specific viral infection models and how to implement the 3Rs

Roberto Plasenzotti

Most zoonotic viral diseases have genetically closely related strains in various mammalian species. In contrast to infecting genetically modified species with human-derived viruses, the use of animal models with the species-specific viruses can lead to a reduction in animal suffering while increasing reproducibility.
Conducting infection experiments does not necessarily mean severe animal suffering, but can be minimized by using the appropriate model and appropriate refinement measures. This presentation builds on the previous two and will provide insight into how the 3Rs can best be considered in these infection models.

Green Toxicology: Making Toxicology a 21st Century, Data Driven Science

Alexandra Maertens

Green Toxicology refers to the use of the tools of toxicology for Green Chemistry.
Central to the goals of Green Chemistry is to design less toxic, more environmentally friendly chemicals - but this can`t happen unless we have a way to conclusively connect chemical structure to the molecular mechanisms of toxicology, something current (animal) assays do poorly, if at all.
Therefore, toxicology will have to evolve into a science that is data-driven and takes advantage of machine learning to map out mechanisms of toxicity in a way that can be more useful for predicting human health hazard.

Refinements through in-vivo Magnetic Resonance Imaging

Clemens Diwoky, Institute of Molecular Biosciences, University of Graz, Graz, Austria

Since its invention in the early 1970s, MR imaging has proven to be the most powerful and versatile medical imaging modality known to date. MR images are rich in spatial detail and exhibit excellent soft tissue contrast. Next to “plain” images, MRI, based fundamentally on the NMR phenomenon, is capable of providing a view of the chemical composition of live tissue. Based on some examples this talk will give an idea how animal studies can be refined by the use of this multiparametric noninvasive imaging technique. Interested users will be given an overview of the general workflow of an MRI study as a guidance and to prevent administrative hurdles in applying MRI to their animal models.

Investigating the Nr4a1 mediated regulation of immune evasion in aggressive lymphoma

Alexander Deutsch

Aggressive lymphomas represent the most common type of lymphoid malignancies with a five-year survival rate of 60%. Despite effective initial treatment, one-third of all patients will experience a relapse, warranting more research to develop novel therapies. We recently detected a significant reduction of nuclear receptor NR4A1 expression in aggressive lymphoma patients that correlated with poor cancer-specific survival. We observed that loss of Nr4a1 leads to an accelerated lymphomagenesis in vivo, concomitant with increased expression of immune checkpoints. Immuno-competent, but not immune-deficient, mice transplanted with Nr4a1-deficient lymphoma cells exhibited rapid lymphoma development, reduced survival, and upregulation of immune checkpoints. To further further dissected the immunoregulatory function of Nr4a1 in aggressive lymphoma., we used OT-1 CD8+ T cells expressing a T cell receptor targeting ovalbumin peptide (OVA) and EμMyc Nr4a1+/+ or EμMyc Nr4a1-/- lymphoma cell lines, which were pulsed with the OVA, in a co-culture cytotoxicity assay. Immune cell mediated lymphoma cell lysis was measured after 4h, 8h, 16h, and 24h, respectively. Interestingly, lymphoma cell killing was diminished in the EμMyc Nr4a1-/- setting after 16h and 24h. Our data suggest that Nr4a1 plays a critical role in regulating the licensing of immune evasion inaggressive lymphomas by regulating the immune checkpoint expression.

Modeling SARS-CoV-2 infection and novel therapeutic targets at barrier sites - An animal-free approach in SARS-CoV-2 research

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Background
Excessive inflammation triggered by a hitherto undescribed mechanism is a hallmark of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections and is associated with enhanced pathogenicity and mortality.

Objective
Complement hyperactivation promotes lung injury and was observed in patients suffering from Middle East respiratory syndrome-related coronavirus, SARS-CoV-1, and SARS-CoV-2 infections. Therefore, we investigated the very first interactions of primary human airway epithelial cells on exposure to SARS-CoV-2 in terms of complement component 3 (C3)-mediated effects.

Methods
For this, we used highly differentiated primary human 3-dimensional tissue models infected with SARS-CoV-2 patient isolates. Upon infection, viral load, viral infectivity, intracellular complement activation, inflammatory mechanisms, and tissue destruction were analyzed by real-time RT-PCR, high content screening, plaque assays, luminex analyses, and transepithelial electrical resistance measurements.

Results
Here, we show that primary normal human bronchial and small airway epithelial cells respond to SARS-CoV-2 infection by an inflated local C3 mobilization. SARS-CoV-2 infection resulted in exaggerated intracellular complement activation and destruction of the epithelial integrity in monolayer cultures of primary human airway cells and highly differentiated, pseudostratified, mucus-producing, ciliated respiratory tissue models. SARS-CoV-2–infected 3-dimensional cultures secreted significantly higher levels of C3a and the proinflammatory cytokines IL-6, monocyte chemoattractant protein 1, IL-1α, and RANTES.

Conclusions
Crucially, we illustrate here for the first time that targeting the anaphylotoxin receptors C3a receptor and C5a receptor in nonimmune respiratory cells can prevent intrinsic lung inflammation and tissue damage. This opens up the exciting possibility in the treatment of COVID-19.

Human induced pluripotent stem cells for toxicological investigations

Paul Jennings

iPSC have great potential for use in tissue regeneration and in vitro toxicological experiments. The ability to expand and biobank iPSC cells from human individuals provides a new cell source, which has the potential to eventually take over from primary cells and cell lines. However, there are also many challenges with iPSC, in terms of good cell culture practices and the development of differentiation procedures. Nevertheless there have been good progress in the field of in vitro toxicology. Some of the experiences from our lab utilising iPSC for nephrotoxicity will be discussed.

Emulating Human Immunity *in vitro*: The Human Artificial Lymph Node Model (HuALN) for Biopharmaceutical Testing and Disease Modelling

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As modern biopharmaceuticals show a very high degree of species-specificity, conventional animal models are inadequate for drug development and to assess efficacy and safety. The Mode of Action (MoA) is complex, so simplified cell culture models using human cells often fail. Customized animal models, e.g. immune deficient, transgenic, and humanized animals as well as xenograft models are big in business but they show limitations in reproducibility and relevance to a certain extent. In particular for the human immune system, drawbacks in the pharmaceutical arena during the last years have raised significant doubts about their value and predictability for assessing immune modulation and immunogenicity. New "humanoid" models are required for new promising pharmaceutical treatments, e.g. by immune modulators, checkpoint inhibitors, and cell- and gene therapeutics.

The dramatically increased animal consumption triggered by customized animal model technologies pushes the ethical concerns on animal testing for pharmaceutical R&D, efficacy and toxicity testing, and risk assessment.

The Human Artificial Lymph Node Model (HuALN) is a micro physiological system (MPS) mimicking immunity in a continuously perfused 3D culture system and suitable for long-term treatment (e.g. 28 d) and repeated dosing. The MPS serves as a human micro-organoid lymph node model for induction or modulation of cellular and humoral immune responses. The implementation of stromal cells improves organoid formation. The HuALN model is designed for testing immunomodulation (e.g. MoA of checkpoint modulators), to assess unwanted immunogenicity reactions (e.g. ADA formation, sensitization) or efficacy of vaccines, adjuvants and formulation. T cell responses and shifts in the TH1/TH2 pathway are continuously monitored by cytokine secretion profiles. The induction of primary humoral responses is demonstrated by B cell activation, plasma cell formation and antibody secretion profiles for IgM and IgG. Cells can be harvested from 3D matrix at the end of the MPS culture time and used for flowcytometric analysis and functional tests, e.g. ELISPOT assays.

By integration of tumour cells or tumour spheroids the HuALN platform is extended towards disease models.

The HuALN model will be introduced, selected results of biopharmaceutical testing will be presented and the opportunities using the HuALN for modelling tumour treatment will be discussed.

Moving Towards Xeno-Free Cell Culture Media: Human Platelet Lysate Outperforms FBS

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The use of fetal bovine serum (FBS) as a cell culture medium is widely spread since it provides a broad spectrum of molecules known to support cell attachment and growth. However, the cruelty of FBS extraction from living bovine foetuses (prohibited in the EU), the lack of traceability of FBS manufacturing and the loose legal regulation of animal welfare and the use of animal-derived products in science have raised strong concerns. Human Platelet Lysate (HPL) is a promising solution for FBS-related concerns. HPL is produced from expired human thrombocyte concentrates, which are clinically tested transfusion products manufactured by certified blood donation centres. Repeated freezing and thawing of thrombocytes result in a highly enriched cocktail of essential growth factors and chemokines. We as PL BioScience have developed a new HPL-based platform called ELAREMTM, which consists of cell culture media that promote growth of many different animal- and humanderived cell types. Our products guarantee high reproducibility due to large batch pools consisting of several hundred platelet donations. Furthermore, our ELAREMTM technology bridges the gaps between academic research, pre-clinical research and cell therapy, as we provide HPL in three different product lines: ELAREMTM Prime, ELAREMTM Perform and ELAREMTM Ultimate.

Strategies for a personalized medicine by using human placenta

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Introduction:
Most biomaterials used for cell culture applications or clinical situations are still extracted from non-human origins, associated with false positive or negative research results and immune reactions in clinical applications.
Placenta, a human waste material, could be used to harvest human extracellular matrix (ECM)-based materials on industrial scales without ethical concerns. The aim of this project was to establish protocols to extract human ECM for applications in tissue engineering.

Methods:
We extracted human non-cellular biomaterials from human placenta using enzymatic-based buffers or non-enzymatic buffers [1,2].

Results:
We observed significant differences between human and non-human biomaterials using different cell types in various 2D and 3D cell culture applications including cell viability, growth rate and phenotype.

Conclusions:
Our studies suggest that the choice of the biomaterials used in tissue engineering significantly influences the outcome of *in vitro* cell studies. Human biomaterials could refine cell culture results which are based on animal-derived materials. The use of new human-derived materials could be an important step for the development of personalized medicine such as *in vitro* cancer or toxicity studies for the future.

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Organ-on-a-chip Technologies: *in vitro veritas*?

Peter Ertl

Organ-on-a-chip systems contain three-dimensional human living cell cultures that are grown in a dynamic microenvironment under controlled measurement conditions. These microphysiological systems allow biological, chemical and physical manipulation and analysis of minimal functional units of human organs and tissues. Consequently, the reliable establishment of human tissue structures on a common chip platform has shown the potential to reduce and replace animal testing in basic and applied research as well as industrial QC measures. Additionally, organ-on-a-chip systems are used to establish personalized disease models with the aim of providing clinical-relevant information from a patient's own cells. In this presentation the current state-of-the-art and selected applications in personalized medicine will be discussed.

Evaluation of inhaled aerosols by physiologically relevant in vitro systems

Fröhlich, E; Meindl, C; Öhlinger, K; Absenger-Novak, M; Kolesnik, T

The lungs are exposed to toxicants in the environment but the lungs are also used as administration route for lung diseases. The respiratory system is the most permeable and vulnerable barrier of the human body. Although in vivo testing is still the gold standard, commonly used laboratory animals have several limitations for the assessment of inhaled aerosols in humans. Therefore, in vitro tests in combination with in silico modeling may have a good chance to replace animal testing in the future. For realistic assessment of pulmonary effects, one has to be aware that the respiratory system consists of two distinct parts, the conductive and the respiratory airways, which differ in cellular composition and morphology and need different in vitro models. Important aspects for the in vitro evaluation of inhalation exposure include culture at the air-liquid interface, presence of mucus or surfactant, co-culture with immune cells, and reconstructed tissues. Ideally, cultures should be stable over several days or weeks to allow repeated exposure to the samples. Examples for the use of various in vitro models will be shown.

Respiratory 3D Barrier Models

Doris Wilflingseder, Institute of Hygiene and Medical Microbiology, Medical University of Innsbruck

Within the workshop ´3D Barrier´ models, a practical overview how to culture two different respiratory 3D barrier models will be given. On the one hand, a perfused, scaffold-based primary model, on the other, how basal-out lung organoids are flipped to an apical-out polarity for immediate apical challenge experiments will be introduced within the workshop.