NLSEV
Netherlands Society for Extracellular Vesicles

Programme book

Seventh annual meeting of the

Netherlands Society for Extracellular Vesicles (NLSEV)

Monday 27th and Tuesday 28th November 2023

HAL4 aan de Maas

Watertorenweg 200, 3063 HA Rotterdam

Sponsoring partners

City of
Rotterdam

Welcome

Dear colleagues,

We are very excited to welcome you at Hal4 aan de Maas in Rotterdam for the seventh annual NLSEV meeting.

Similar to last year, we are proud to present to you a two-day program that is filled with two keynote lectures, 17 selected oral abstract presentations and more than 40 posters covering the large variation of EV research. The detailed program and the abstracts of the presentations and posters can be found in this abstract book. We want to encourage you to visit the posters at both floors of the venue and have lively discussions with the poster presenters during the coffee breaks and posterviewing sessions. The program also includes plenty of opportunities for networking and social interactions. This is emphasized by the Networking Event in EAU Lounge at Monday evening with a walking diner and music.

We are enormously grateful for the support by the multitude of loyal Sponsoring Partners.The commercial partners have been and will be incredibly valuable within the field of EV research in the Netherlands, with regards to novel technologies for EV isolation and characterization but also at the stage of application of EVs in diagnostics or therapies. We highly encourage you to reach out to the sponsoring partners to gain insight on their current states of the art technologies and services and to explore potential synergy or collaboration. Also many thanks to the City of Rotterdam with the additional support to enable this meeting, in particular to realize a Networking Event with beautiful view on the city and the river Maas, a perfect location to mingle and to get to know each other even better.

We wish you a great and fruitful meeting, with lots of enjoyable moments, together with your fellow EV community members!

The Local Organization Committee;
Martin van Royen, Department of Pathology, Erasmus Medical Center
Karin Boer, Department of Internal Medicine, Erasmus Medical Center
Guido Jenster, Department of Urology, Erasmus Medical Center
Marie-José Goumans, Department of Cell and Chemical Biology, Leiden University Medical Center
Jeroen de Vrij, Department of Neurosurgery, Erasmus Medical Center

NLSEV
Netherlands Society for Extracellular Vesicles

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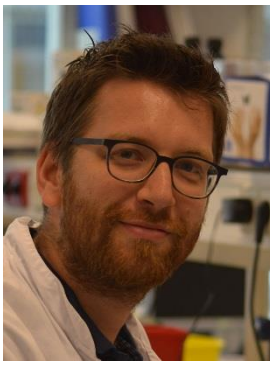

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Marie-José Goumans

Jeroen de Vrij

Practical information

Meeting venue address:

Hal4 aan de Maas

Watertorenweg 200

3063 HA Rotterdam

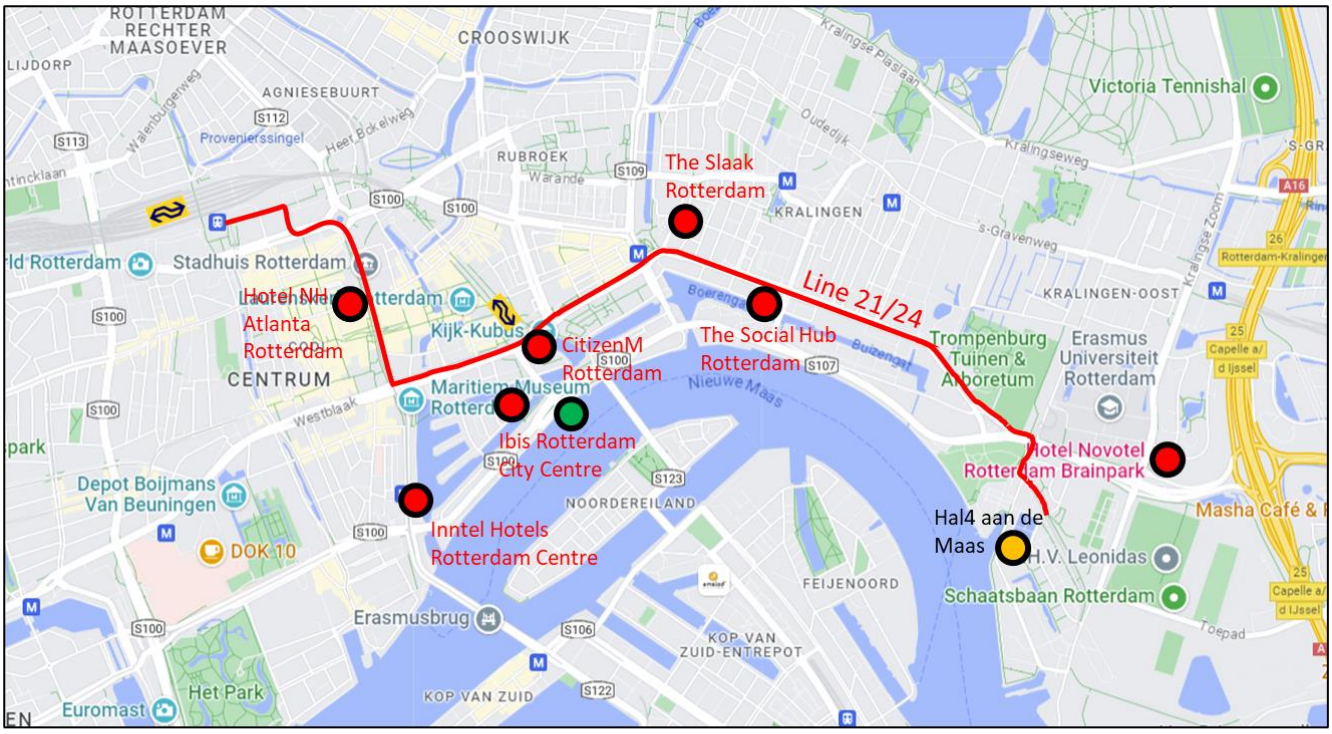
Parking: paid parking (via app) is available close to the venue.

Network event venue address:

EAU Lounge

Boompjes 750, 3011 XZ Rotterdam

Paid Parking: Noordereiland, Oude Haven and parking Markthal



Practical information

Public transportation:

RET tram line 21/24 - direction De Esch
(red line op the map)

Meeting venue:
Hal 4 aan de Maas/meeting venue is a 3 min walk from RET tram line stop Lage Filterweg (~20 min from Rotterdam Centraal)

Networking event venue:
EAU Lounge is a 5 min walk from RET tram line stop Station Blaak

Programme

Monday, November 27th

12.30 - 13.30 Registration & Poster Mounting

13.30 - 13.45 Welcome by Esther Nolte-'t Hoen and the Local Organising Committee

13.45 - 14.30 Keynote lecture

Chairs: Esther Nolte 't Hoen and Jelle van den Bor

Roosmarijn Vandenbroucke, *Ghent University and VIB, Ghent*
The importance of (bacterial) extracellular vesicles in the communication between periphery and brain

14.30 - 15.00 Selected presentations: EV biogenesis I

O1: Misko Bobeldijk *Utrecht University, Utrecht, The Netherlands*
Extracellular vesicles in a FLASH; live-tracking of EV-cargo proteins from the ER to their destination

O2: Anusca Rader *Amsterdam UMC, Amsterdam, The Netherlands*
Dengue virus exploits autophagy vesicles and secretory pathways to promote transmission by human dendritic cells

15.00 – 15.30 Coffee break & Poster viewing

15.30 – 16.15 Selected presentations: EV biogenesis II

Chairs: Kasper Rouschop and Britta Bettin

O3: Weicheng Xu *Erasmus Medical Center, Rotterdam, The Netherlands*
The effect of immune modulation on the release of extracellular vesicles

O4: Chao Li *Leiden University Medical Center, Leiden, The Netherlands*
TGF-β modulate the secretion of extracellular vesicles and the protein cargo sorting by downregulating RAB27B expression in breast cancer.

O5: Anna George *Utrecht University, Utrecht, The Netherlands*
Taking Control of Exosome Secretion

Programme

16.15 - 16.25 Sponsored talks

S1: Daniel Lueert *Beckman Coulter*
BEC LS Solutions for EV research, an update

16.25 - 17.25 Coffee break & Poster viewing

17.25 - 17.40 Updates from the NLSEV board

17.40 – 18.10 Selected presentations: EV Biomarkers
Chairs: Rienk Nieuwland and Augustin Enciso Martinez

O6: Liang Wu *Erasmus Medical Center, Rotterdam, The Netherlands*
Dynamic Release of Kidney-Derived Urinary Extracellular Vesicles Post-Transplantation

O7: Chantal Scheepbouwer *Amsterdam UMC, Amsterdam, The Netherlands*
Profiling of extracellular small RNA reveals insight into tRNA CCA tail integrity

18.10 – 18.20 Sponsored talks

S2: Stephane Mazlan *IZON*
Increasing small particle recovery and extracellular vesicle (EV) isolate purity with size exclusion chromatography

18.20 – 19.30 Poster Meet & Greet with Drinks & Snacks

20.00 – 23.00 Network event with diner at Eau Lounge, Boompjes 750 Rotterdam

Programme

Tuesday, November 28th

08.30 - 09.00 Arrival/Registration and coffee

09.00 - 09.15 EV Delta Presentation

09.15 - 09.45 Selected presentations: EVs and EV cargo delivery I
Chairs: Rubina Baglio and Lei Zhu

O8: Maria Laura *UMC Utrecht, Utrecht, The Netherlands*
Unravelling molecular drivers of extracellular vesicle-mediated cargo transfer

O9: Xiaoqian Gong *Utrecht University, Utrecht, The Netherlands*
Infectious viral genomes can be packaged and delivered by extracellular vesicles with or without viral capsids

09.45 – 09.55 Sponsored talk

S3: Evgeniya Pechnikova *DENSsolutions*
Controlling the biochemistry on-the-fly and visualizing reaction dynamics during in-situ Liquid Phase TEM: A strong tool for biopharmaceutical development

09.55 - 10.55 Coffee break & Poster viewing

10.55 -11.40 Keynote lecture
Chairs: Bas van Balkom and Ardalan Mansouri

Stefan Momma *Johann Wolfgang Goethe University, Frankfurt am Main*
Inter-organ and microbe-host communication

11.40 -12.10 Selected presentations: EVs and EV cargo delivery II

O10: Xin Xin *Maastricht University, Maastricht, The Netherlands*
Optimization of purification and fluorescent labeling of platelet-derived extracellular vesicles for therapeutic drug delivery

O11: Willemijn de Voogt *UMC Utrecht, Utrecht, The Netherlands*
Tracing extracellular vesicle subpopulations using HaloTag fusion proteins

12.10-13.20 Lunch break & Poster Viewing

Programme

13.20 – 14.05 Selected presentations: EV in cancer
Chairs: Frederick Verweij and Liang Wu

O12: Steven Wang *Amsterdam UMC, Amsterdam, The Netherlands*
Vesicle RNA Profiling and Deconvolution for Outcome Prediction in Aggressive B-Cell Lymphoma

O13: Barbara Adem *University, Utrecht, The Netherlands*
CD63 positive extracellular vesicles define a plastic network of communication in pancreatic cancer

O14: Crescenzo Massaro *Amsterdam UMC, Amsterdam, Utrecht, The Netherlands*
Tumor-secreted extracellular vesicles impair bone cancer therapy response by shaping the bone marrow immune environment

14.05 – 14.15 Sponsored talk

S4: Ingrid Bloss *Particle Metrix*
NTA with ZetaView - More than just size and concentration

14.15 - 14.45 Coffee break & Poster Viewing

14.45 - 15.30 Selected presentations: EV dectection technology
Chairs: Pieter Vader and Wouter Woud

O15: Britta Bettin *Amsterdam UMC, Amsterdam, The Netherlands*
Global inter-laboratory comparison study to standardize EV concentration measurements between 39 flow cytometers

O16: Sasidharan Sajitha *University Groningen, Groningen, The Netherlands*
Mechanical Characterization of Extracellular Vesicles using AFM-based Nanoindentation Methods

O17: Mendel Engelaer *Amsterdam UMC, Amsterdam, The Netherlands*
A new practical procedure to set and report the trigger threshold of flow cytometers

15.30 - 16.00 NLSEV awards and closing

Keynote Speakers

Oral presentations – Keynote speaker

Prof Dr Roosmarijn Vandenbroucke

Ghent University and VIB, Ghent, Belgium

Prof. Dr. Vandenbroucke earned her Master's degree in Biotechnology and completed her PhD in Pharmaceutical Sciences in 2008 at Ghent University, specializing in gene therapy. Following her postdoctoral research, she developed a keen interest in the blood-CSF interface situated at the choroid plexus. In 2015, she established her independent research lab at Ghent University and at VIB (Belgium) in 2018. Leading the 'Barriers in Inflammation' team, her research focuses on unraveling the complexities of the blood-brain interface, exploring the significance of the gut-brain axis in neurological disorders, and improving drug delivery to the brain. Notably, she recently received an ERC CoG grant.

Keynote Speakers

Oral presentations – Keynote speaker

Prof Dr Stefan Momma

Johann Wolfgang Goethe University, Frankfurt am Main

Stefan Momma is a principal investigator in the Neurological Institute of the University Hospital Frankfurt am Main, Germany. He has a broad background in the neurosciences with a focus on neural stem cells and adult neurogenesis, neurodegenerative diseases, as well as malignancies of the brain. Departing from his main research area into questions of how the brain interacts with other organs, he developed an interest in the topic of extracellular vesicles (EV). Here, he developed a transgenic mouse model that can be used to trace EV signaling between cells, thereby providing the first evidence for functional RNA or protein transfer by EVs in vivo and unveiling some of the parameters that predict their uptake. More recently, he extended this approach to study interspecies communication by EVs, notably between the gut microbiome and host organs, including the brain.

Selected Oral Presentations

NLSEV2023-O1 Misko Bobeldijk

EVs in a FLASH; live-tracking of EV-cargo proteins from the ER to their destination

Misko Bobeldijk [1, 2], Lenny Droesen [1], Jelle van den Bor [1], Elly Soltani [1], Frederik Verweij [1, 2]
1: Department of Cell Biology, Neurobiology, and Biophysics, Utrecht University, 2: Centre for Living Technologies, Eindhoven, Wageningen, and Utrecht.

INTRODUCTION: Extracellular vesicles (EVs) are small vesicles secreted by all cell types that play a crucial role in intercellular communication by engaging receptor molecules on target cells. Changes in EV secretion, cargo composition, and their impact on disease progression underscore the need to understand EV biogenesis and cargo loading, especially under pathological conditions. However, a comprehensive tool for unaltered, endogenous visualization of EV-cargo protein trafficking within the secreting cell is currently lacking. This study presents a novel method for pulse-chase labeling and tracking EV-cargo proteins, starting from their synthesis in the endoplasmic reticulum (ER). This approach provides insights into the trafficking of CD63, a common EV marker, VEGFA, a recently identified EV protein corona constituent, and TSPAN2 and 3 which are newly identified EV subtype markers.

METHODS: We developed a split-HALO-tag system to investigate the intracellular trafficking of de novo-synthesized CD63, VEGFA, TSPAN2, and TSPAN3. One tag was linked to the protein of interest (POI), while the other was introduced into the ER with an IL2 targeting motif. This setup allowed us to visualize POI trafficking and loading into EVs using fixed-cell imaging, live-cell spinning disc, and total internal resonance fluorescence microscopy (TIRFM).

RESULTS: Our SplitHALO system enabled specific labeling of ER-resident CD63, VEGFA, TSPAN2, and TSPAN3 populations without the need for temperature, protein, or drug-mediated ER retention. This approach allowed us to track the spatiotemporal dynamics of their trafficking from the ER to various cellular compartments. Additionally, dual-color TIRFM revealed that a substantial portion of newly synthesized CD63 directly enters the secretory multivesicular body (MVB) pathway after leaving the ER.

CONCLUSIONS: In this study, we introduce a novel tool for pulse-labeling ER-resident soluble and transmembrane proteins, eliminating the necessity for prior ER retention. This advancement enhances the accuracy of assessing their real-time trafficking dynamics. Using this innovative tool, we uncovered that de novo-synthesized CD63 follows unknown pathways to reach fusogenic MVBs, a previously unreported phenomenon. Our ongoing work aims to extend this analysis to other known EV-cargo proteins, providing further insights into EV biology and its relevance to diseases.

Selected Oral Presentations

NLSEV2023-O2 Anusca G. Rader

Dengue virus exploits autophagy vesicles and secretory pathways to promote transmission by human dendritic cells

Anusca G. Rader [1,2,3], Alexandra P.M. Cloherty [1,2], Kharishma S. Patel [1,2], Tracy-Jane T.H.D. Eisden [1,2,4], Sterre van Piggelen [1,2], Renée R.C.E. Schreurs [1,2,3], Carla M.S. Ribeiro [1,2,3]

1: Department of Experimental Immunology, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands, 2: Infectious Diseases & Inflammatory Diseases Programs, Amsterdam institute for Infection & Immunity, Amsterdam, The Netherlands, 3: Amsterdam Gastroenterology & Metabolism, University of Amsterdam, Amsterdam, The Netherlands, 4: Department of Medical Oncology, Cancer Center Amsterdam, Amsterdam UMC, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands.

INTRODUCTION: Dengue virus (DENV), transmitted by infected mosquitoes, is a major public health concern, with half the world's population at risk for infection. Recent decades have increasing incidence of dengue-associated disease alongside growing frequency of outbreaks. Although promising progress has been made in anti-DENV immunizations, post-infection treatment remains limited. Dendritic cells (DCs) are amongst the first human cells to encounter DENV upon injection into the skin, and thereafter promote systemic viral dissemination to additional target cells. Autophagy is an intracellular vesicle trafficking pathway, and has been implicated in DENV disease pathogenesis. However, the function of autophagy pathways in DENV infection and dissemination by human primary DCs remains poorly understood. Herein, we demonstrate that autophagy mechanisms and extracellular vesicles (EVs) have a pro-viral role in DENV dissemination by DCs.

METHODS: RNA interference was employed to investigate the role of autophagy machinery on DENV replication in primary human DCs. Autophagy activity and DENV infection and transmission were determined by flow cytometry and confocal microscopy. DC-derived EVs were purified by immunomagnetic positive selection. Immunophenotypic profiling of EVs, including expression of autophagy markers, was performed using imaging flow cytometry analyses.

RESULTS: We show that DENV exploits early-stage autophagy machinery to establish infection in primary human DCs. DENV replication induced autophagosome formation in DCs, and intrinsically-heightened autophagosome biogenesis correlated with relatively higher rates of DC susceptibility to DENV. Furthermore, our data demonstrates targeting of viral replication intermediates to autophagosomes, while productive DENV infection introduces a block at the late degradative stages of autophagy in infected DCs but not in uninfected bystander cells. Notably, we identify for the first time that approximately one-fourth of DC-derived CD9/CD81/CD63+ EVs co-express canonical autophagy marker LC3, and demonstrate that DC-derived EVs are an alternative, cell-free mechanism by which DCs promote DENV dissemination to additional target sites.

DISCUSSION: Taken together, our study highlights intersections between autophagy and secretory pathways, and puts forward autophagosome generation and infectious EV release as host determinants of DC-mediated DENV infection in humans. Host-directed therapeutics targeting autophagy and exocytosis mechanisms thus have promising potential to enhance DC-driven resistance to DENV acquisition and thereby limit viral dissemination.

Selected Oral Presentations

NLSEV2023-O3 Weicheng Xu

The effect of immune modulation on the release of extracellular vesicles

Weicheng Xu [1], Liang Wu [1], Marjolein Dieterich [1], Dennis A. Hesselink [1], Karin Boer [1], Carla C. Baan [1]
1: Erasmus MC Transplant Institute, University Medical Center Rotterdam, Department of Internal Medicine, Division of Nephrology and Transplantation, Rotterdam, The Netherlands.

INTRODUCTION: After kidney transplantation, recipients receive immunosuppressive treatment to prevent rejection. Extracellular vesicles (EVs) play a critical role in regulating post-transplant immune response by conveying antigens, cytokines, and mRNAs between transplanted kidney and recipient immune cells to influence the alloantigen presentation and cell-to-cell communication. However, The effect of immunosuppressants on EV release is unknown. We examined the effects of immunosuppressants (tacrolimus, prednisolone, and mycophenolate acid (MPA)) on the release of EVs, including their size and phenotypic features.

METHODS: THP-1 cells (monocytes) were cultured in the absence and presence of therapeutic concentrations of tacrolimus (10ng/ml), prednisolone (1ug/ml), and MPA (1ug/ml). To examine the effect of immune activation, THP-1 cells were exposed to Interferon (IFN) γ (50ng/ml). Particle size and concentration were measured using nanoparticle tracking analysis (NTA). An isolation-free imaging flow cytometry (IFCM) protocol was used to characterize and quantify the EV subpopulations (CD9, CD63, CD81) and the immune activation marker HLA-class I.

RESULTS: None of the tested immunosuppressive agents affected EV size and concentration. IFCM showed that in the absence of immunosuppressive drugs, THP-1–derived EV expressed either CD9 (29%), CD63 (30%), or CD81 (41%) which was comparable to the proportion of CD9, CD63, and CD81 EVs when measured in the presence of tacrolimus, prednisolone or MPA. Regardless of the use of the immunosuppressants, the percentage of HLA-class I expression was <26% for all EV (CD9, CD81, CD63) subpopulations. In addition, IFN-γ treatment reduced EV release by 59% after 48 hours, while EV size was not affected. After IFN-γ treatment, the majority of these EVs expressed CD63 (43%) with an upregulated proportion expressing HLA-class I (46%). Moreover, under these immune-activated conditions, the addition of tacrolimus, prednisolone, or MPA did not alter EV release, size, and phenotype.

CONCLUSION: Immunosuppressive agents (tacrolimus, prednisolone, and MPA) do not affect EV release by monocytes, while IFN-γ inhibits EV release and upregulates EV-HLA expression.

Selected Oral Presentations

NLSEV2023-O4 Chao Li

TGF-β modulate the secretion of extracellular vesicles and the protein cargo sorting by downregulating RAB27B expression in breast cancer

Chao Li [1], Agustin Enciso Martinez [1,2], Peter Ten Dijke [1]

1: Oncode Institute and Department of Cell and Chemical Biology, Leiden University Medical Center, Leiden, The Netherlands,
2: Amsterdam Vesicle Center, Biomedical Engineering and Physics, Amsterdam University Medical Center, The Netherlands.

INTRODUCTION: TGF-β signaling has important role in cancer development. The activated TGF-β signaling can promote cancer cell invasion and metastasis, and also shape the tumor microenvironment (TME) by affecting immune cells, cancer-associated fibroblasts (CAFs), endothelial cells, and other stroma cell types. Small extracellular vesicles (sEVs) are versatile messengers in TME which play a key role in intercellular communications by delivering heterogeneous cargos. However, it is unclear whether TGF-β can affect EVs secretion from cancer cells and how TGF-β regulates the TME by altering the cargos of cancer cell-derived EVs. In this study, we revealed that TGF-β affects secretion and composition of EVs by regulating RAB27B expression, leading to a stronger immunosuppressive role of EVs.

METHODS: SEVs were isolated using ultrafiltration followed by size exclusion chromatography (SEC). Microfluidic resistive pulse sensing (MRPS) was performed to obtain accurate concentration of EVs. The secretion of EVs was also visualized by CD63-pHluorin reporter using total internal reflection microscopy (TIRF). 4D label-free quantitative proteomics was performed to compare how TGF-β affects the protein cargo sorting into EVs based on equal number of EVs measured by MRPS. Human CD8+ T cells were used as recipient cells of cancer cell-derived EVs to examine the immunosuppressive functions of EVs.

RESULTS: The secretion of EVs is downregulated by TGF-β treatment in MDA-MB-231 cells. TGF-β- induced SMAD complexes can inhibit the transcription of RAB27B in both direct and indirect ways, which leads to the reduction of mRNA and protein level of RAB27B. The decreased RAB27B reduced the events of the docking of multivesicular bodies (MVBs) to the membrane, and then the secretion of EVs decreased. However, the decrease of RAB27B can elevate the amount of protein cargos sorted into EVs and change of composition of them. When the CD8+ T cells were exposed to equal numbers of MDA-MB-231- derived EVs, the TGF-β induced-EVs showed stronger immunosuppressive effects to the CD8+ T cells.

CONCLUSIONS: TGF-β enhanced the immunosuppressive functions of breast cancer cells-derived EVs by reducing the EV number and increasing the amount of protein cargos mediated by the reduction of RAB27B.

Selected Oral Presentations

NLSEV2023-O5 Anna E. George

Taking control of exosome secretion

Anna E. George [1,2], Lara Oprešnik [1], Lukas C. Kapitein [1,2], Frederik Verweij [1,2]
1. Department of Cell Biology, Neurobiology and Biophysics, Utrecht University, 2. Center for Living technologies, The Netherlands.

INTRODUCTION: Exosomes are a sub-population of Extracellular Vesicles (EVs) secreted from a cell when Multivesicular Bodies (MVBs), instead of fusing with lysosomes, travel to the plus end of microtubules (MT) to fuse with the Plasma Membrane (PM). The wide variety of functions attributed to EVs is supported by an equally extensive diversity of EV subtypes, including a wide range of MVB subpopulations. However, current tools to control exosome secretion using genetic or drug based interference with regulatory proteins suffer from unwanted side-effects and cannot target specific subpopulations. This represents an enormous challenge to understand endogenous EV function and link a precise function to a precise EV subtype.
To start addressing this, we developed chemo- and optogenetic tools to precisely control the intracellular fate of MVBs, thereby taking control of exosome secretion in a tetraspanin (TSPAN)-selective manner.

METHODS: We developed a heterodimerisation-based coupling of endosomal maturation- and trafficking-associated factors to MVBs by optogenetic- and chemogenetic approaches, that we validated with Total Internal Reflection Fluorescence (TIRF) and confocal microscopy techniques.

RESULTS: To promote exosome secretion, we repositioned CD63-MVBs to the cell periphery by coupling them to plus end directed MT motor proteins, resulting in a specific increase of CD63 positive MVB/PM fusion. Conversely, to reduce exosome secretion, we hypothesized that rather than merely preventing PM fusion, potentially inducing endosomal stress, redirecting MVBs from the secretory pathway towards fusion with lysosomes would provide the ideal strategy. For this, we optogenetically coupled key regulators of MVB-lysosome fusion to CD63-MVBs, and confirmed their increased overlap with lysosomal markers SirLyso and LAMTOR4 upon light activation. Consistent with this, we found selective reduction in CD63-MVB/PM fusion.

CONCLUSIONS:
Here we demonstrate how we can specifically modulate the fate of MVB-subpopulations using opto- and chemogenetic heterodimerization approaches, thereby taking control of exosome secretion. Currently, we are further validating this tool and expanding it to different MVB subtypes (e.g. CD81 and CD9). This study thereby opens the possibility of controlling endogenous EV secretion in vitro and in vivo in a tissue specific manner, allowing us to start unravelling the (patho)physiological functions of EVs with an hitherto inaccessible level of precision.

Selected Oral Presentations

NLSEV2023-O6 Liang Wu

Dynamic release of kidney-derived urinary extracellular vesicles post-transplantation

Liang Wu [1,2], Weicheng Xu [2], Derek Reijerkerk [2], Dennis A. Hesselink [2], Carla C. Baan [2], Karin Boer [2]
1: Department of Nephrology, The First Affiliated Hospital of Shaoyang University, Shaoyang, Hunan, China, 2: Erasmus MC Transplant Institute, University Medical Center Rotterdam, Department of Internal Medicine, Division of Nephrology and Transplantation, Rotterdam, the Netherlands.

INTRODUCTION: Kidney transplantation is the preferred treatment for patients with end-stage renal disease (ESRD). The conventional method for diagnosing post-transplant rejection, a renal biopsy, is invasive and restricted due to bleeding and infection risks. Novel, preferably non-invasive markers are needed to indicate allograft status, including rejection. Urinary extracellular vesicles (uEVs) are promising candidates, but the dynamics of uEV release from transplanted kidneys are poorly known. This study investigates the presence of kidney-derived uEVs at various time points post-transplantation.

METHODS: Urine was collected from 45 donor-recipient pairs before transplantation and from recipients on day 3 (D3), day 7 (D7), and month 6 (M6) post-transplant. The collected urine was centrifuged to remove cells, and uEVs were stained with the EV-marker CD63 individually or a combination of CD63 with a kidney-specific marker, aquaporin 2 (AQP2; collecting duct), or podocalyxin (PODXL; podocytes) without uEV isolation. uEVs were quantified using imaging flow cytometry with detergent treatment and isotype staining as controls.

RESULTS: Of the CD63+ uEVs from donor urine, 4.2 [0.5 – 14.8]% were AQP2+, and 6.4 [1.5 – 14.2]% were PODXL+. Positive correlations were observed between kidney function (estimated glomerular filtration rate) and these kidney-derived uEV percentages, representing $R2 = 0.15$ ($p = 0.075$; AQP2+) and $R2 = 0.20$ ($p = 0.039$; PODXL+). Compared with donor urine, pre-transplant recipient urine displayed minimal levels of kidney-derived uEVs, with 1.3 [0.4 – 2.6]% ($p = 0.13$; CD63+AQP2+) and 1.5 [0.9 – 3.9]% ($p = 0.04$; CD63+PODXL+). These recipient levels remained unchanged on D3 post-transplant (both $p = 0.99$) but significantly increased on D7 to 8.4 [3.9 – 18.4]% ($p < 0.001$; CD63+AQP2+) and 8.2 [3.1 – 15.4]% ($p < 0.001$; CD63+PODXL+) which persisted until M6.

CONCLUSION: Before kidney transplantation, ESRD patients exhibit lower kidney-derived uEV proportions compared to healthy donors. After transplantation, kidney allografts do not demonstrate immediate uEV recovery on D3, but from D7 onwards, kidney-specific uEVs are released. The dynamic release of kidney-specific uEVs might suggest their potential as markers for assessing allograft status, including delayed allograft function and rejection.

Selected Oral Presentations

NLSEV2023-O7 Chantal Scheepbouwer

Profiling of extracellular small RNA reveals insight into tRNA CCA tail integrity

Chantal Scheepbouwer [1,2,3], Ernesto Aparicio-Puerta [4], Monique A.J. van Eijndhoven [3,5], Cristina Gómez-Martin [3,5], Esther E.E. Drees [3,5,6], Leontien Bosch [3,5], Josée M. Zijlstra [5,6], Thomas Wurdinger [1], Daphne de Jong [3,5], Michael Hackenberg [4,7-9], Alan Gerber [1,2], D. Michiel Pegtel [3,5]

1: Department of Neurosurgery, Cancer Center Amsterdam, Amsterdam University Medical Center, VU University, Amsterdam, Netherlands, 2: Cancer Center Amsterdam, Cancer Biology, Amsterdam, Netherlands, 3: Department of Pathology, Cancer Center Amsterdam, Amsterdam University Medical Center, VU University, Amsterdam, Netherlands, 4: Genetics Department, Faculty of Science, Universidad de Granada, Granada, Spain, 5: Cancer Center Amsterdam, Imaging and Biomarkers, Amsterdam, Netherlands, 6: Department of Hematology, Cancer Center Amsterdam, Amsterdam University Medical Center, VU University, Amsterdam, Netherlands, 7: Bioinformatics Laboratory, Biomedical Research Centre (CIBM), Biotechnology Institute, PTS, Granada, Spain, 8: Excellence Research Unit "Modeling Nature" (MNat), University of Granada, Granada, Spain, 9: Instituto de Investigación Biosanitaria ibs.GRANADA, University Hospitals of Granada-University of Granada, Spain.

The content of bulk extracellular vesicles (EVs) isolated from cell culture supernatant reflect, to a large extent, that of the donor cells. While EVs were previously shown to contain functional messenger,- and microRNAs, there are conflicting observations on the nature of the transfer RNA (tRNA) content of EVs. In this study, we determined the small RNA content within EVs using the ALL-tRNAseq sequencing approach that, unlike standard approaches, allows the detection of a diversity of highly structured full-length RNA species, including tRNA, small nucleolar RNA (snoRNA) and Y RNA. We showed, in multiple cell types, that among the small RNA classes in EVs, full-length tRNAs are by far the most abundant group. Importantly, we uncovered that tRNAs exist in EVs mainly as non-functional full-length molecules lacking their functional CCA-tails required for amino acid loading. Although these tRNAs cannot be used in translation, we demonstrated that they are fully modified molecules, highly resembling those of cellular tRNAs. These tRNAs are thus likely originating from previously functional cytoplasmic tRNAs, rather than newly synthesized tRNAs that are removed from the cell as a result of improper processing. Remarkably, we could recapitulate these distinct 3' terminal patterns of tRNAs in circulating plasma EVs obtained from individuals with classical Hodgkin lymphoma (cHL), which seemed to be modulated in response to treatment. Taken together, these results emphasize a significant and overlooked aspect of the small RNA composition within EVs, which could serve as reference for future investigations into their function and as disease biomarkers.

Selected Oral Presentations

NLSEV2023-O8 Maria Laura Tognoli

Unravelling molecular drivers of extracellular vesicle-mediated cargo transfer

Maria Laura Tognoli [1], Julia Dancourt [2], Emeline Bonsergent [2], Roberta Palmulli [3], Olivier G. de Jong [4], Andre Görgens [5], Samir El Andaloussi [5], Guillaume Van Niel [3], Eric Rubinstein [6], Gregory Lavieu [2], Pieter Vader [1,7]

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Extracellular vesicles (EVs) are thought to mediate intercellular communication by transferring bioactive cargo from donor to acceptor cells. The EV content delivery process within acceptor cells, however, is still poorly characterized and the molecular players at the donor EV/acceptor cell interface have not been elucidated.
Here, we set out to identify critical surface proteins on EVs that mediate functional cargo transfer in recipient cells and understand how these proteins affect different steps of the delivery process. First, we mapped the surfaceome of isolated EVs by coupling high purification steps (size exclusion chromatography followed by density gradient ultracentrifugation) and mass spectrometry. We identified approximately 200 proteins that decorate the membrane of MDA-MB-231 and hTERT-MSC derived EVs. Next, we performed a siRNA-based screen targeting the aforementioned genes to evaluate their involvement in EV-mediated (RNA) cargo transfer in a co-culture system. We are now validating the screen hits, via chemical or antibody-based inhibition, and evaluating their involvement in the EV content delivery process with in-house designed assays. Among the surface proteins identified via mass spectrometry, tetraspanins CD63 and CD9 have been previously implicated in EV mediated cargo transfer. However, using two different reporter assays (CROSS-FIRE and Nanoluciferase assay) we found that both CD63 and CD9 are dispensable for EV mediated cargo transfer, both on the donor EV's and on the acceptor cell's side. Furthermore, additional data similarly supports dispensability for CD81 in this process.

In conclusion, we have identified EV surface proteins and, through a RNA interference based screen, we discovered both potential novel regulators, currently under validation, and non-regulators of the EV cargo transfer process. This study has the potential to answer outstanding questions in EV biology on the mechanisms of EV-mediated content delivery, as well as advance the drug delivery field by providing native cargo delivery enhancers for testing in therapeutic formulations.

Selected Oral Presentations

NLSEV2023-O9 Xiaoqian Gong

Infectious viral genomes can be packaged and delivered by EVs with or without viral capsids

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INTRODUCTION: Non-enveloped (naked) viruses can be packaged and released by infected cells inside extracellular vesicles (EVs). The EV membrane protects enclosed virus particles against neutralizing antibodies, thereby facilitating virus spread. We previously discovered that Encephalomyocarditis virus (EMCV) and Coxsackie virus B3 (CVB3), which are non-enveloped, single-strand RNA viruses of the picornavirus family, are released via EVs in the pre-lytic stage of infection. As naked virions, the infectious genomic RNA of these viruses is packaged in a protein capsid. The capsid functions to protect the genomic RNA, interact with host cell virus receptors, and deliver the virus RNA into host cells. We here investigated whether these capsid proteins were necessary for EV-mediated virus spreading, or whether infectious viral genomes not surrounded by capsids could also be packaged and spread via EVs.

METHODS: Cells were infected with genetically modified CVB3 that does not encode for capsid proteins. EVs from infected cells were assessed for quantifying CVB3 RNAs using RT-qPCR and infectivity by CCID50. To compare the CVB3 RNA packaging efficiency and infectivity in the absence and presence of capsid proteins, cells constitutively expressing CVB3 capsid proteins were used.

RESULTS: EVs released by infected cells can transmit infection by transferring viral RNAs without capsids. Both the efficiency of viral RNA packaging into EVs and the infectivity of these particles were substantially lower than those in the presence of capsid proteins. In addition, we observed that the strong induction of EV release normally observed upon CVB3 infection did not occur in the absence of capsids.

CONCLUSION: Encapsidation of the CVB3 RNAs strongly enhances the release of infectious viral RNAs via EVs, the delivery of these RNAs into target cells, and propagation of the infection. Yet, infectious EVs containing only viral RNA can also be formed. Such EVs may enhance spreading of the infection to target cells that lack virus receptors. In future research we will further explore compositional and functional differences between infectious EVs with and without capsids released during wild type CVB3 infections.

Selected Oral Presentations

NLSEV2023-O10 X. Xin

Optimization of purification and fluorescent labeling of platelet-derived extracellular vesicles for therapeutic drug delivery

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INTRODUCTION: Platelet-derived extracellular vesicles (PEVs) are mediators of cell communication and have platelet-mimicking properties such as reduced uptake of macrophage-like cells and their ability to target damaged blood vessels, highlighting their advantages as drug delivery vehicles. However, due to the strict and limited storage conditions of platelets, recently research on replacing fresh platelets with freeze-dried platelets has gained attention. In addition, labeling of PEVs is a widely used method for understanding their cellular targeting and biodistribution. However, fluorescent labeling methods and their effects on PEVs have hardly been investigated so far. The objectives of this study were to investigate whether freeze-dried platelets can replace fresh platelets as a source of PEVs, to find an appropriate fluorescent dye for labeling PEVs and to explore whether vascular endothelial cells can take up PEVs.

METHODS: The size distribution and structure of the PEVs were measured by nanoparticle tracking analysis (NTA) and transmission electron microscopy. The protein biomarkers of the PEVs were detected by Western blot and flow cytometry (FC). The effect of various fluorescent dyes (lipophilic fluorescent and lumen-binding dyes) on the labeling efficiency and size of PEVs was systematically evaluated using NTA and FC. To investigate the ability of endothelial cells to take up PEVs, EA.hy926 cells were cultured at 37°C ,treated with labeled PEVs, and subsequently analysed by fluorescence microscopy and FC.

RESULTS: The results showed that PEVs from freeze-dried platelets were comparable to those from fresh platelets in terms of size distribution, structure, and protein content. NTA and FC demonstrated that lipophilic fluorescent dye 3,3'-Dihexyloxacarbocyanine Iodide (DioC6) had the highest PEVs labeling efficiency and the smallest change in PEVs size after labeling. Moreover, fluorescence microscopy and FC clearly showed that the labeled PEVs were taken up by the endothelial cells.

CONCLUSION: This study showed that freeze-dried platelets could be an alternative to fresh platelets as a source of PEVs. In addition, suitable fluorescent dyes for labeling PEVs were identified and it appeared that PEVs can be taken up by endothelial cells. This may be very useful opening new horizons on diagnostic and therapeutic protocols exploiting PEVs.

Selected Oral Presentations

NLSEV2023-O11 Willemijn S. de Voogt

Tracing extracellular vesicle subpopulations using HaloTag fusion proteins

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INTRODUCTION: Extracellular vesicles (EVs) represent a heterogeneous population of endogenous intercellular delivery vehicles for biomolecules including proteins, nucleic acids and lipids. EVs intrinsically possess properties such as low immunogenicity and high cargo delivery efficiency that make them attractive carrier systems for delivery of biological therapeutics. The main challenge in employing EVs for drug delivery, however, is a lack of understanding of the intracellular trafficking and processing mechanisms of different EV subpopulations within recipient cells. Here, we show that CD63 and ITGA2 define two distinct subpopulations of EVs, which we individually trace in recipient cells using HaloTag fusion proteins compatible with fluorophores with high quantum yield.

METHODS: MDA-MB-231 EVs were captured on poly-L-lysine coated coverslips. dSTORM imaging of CD63 and ITGA2 on EVs was performed using a Nanoimager-S (ONI). HDBSCAN clustering was performed to quantify coincidence of CD63 and ITGA2 on single EVs. EVs immunoprecipitated using CD63 and ITGA2 antibody-coated protein G Dynabeads were characterized by WB. CD63 and ITGA2 fused to HaloTags were expressed in EV donor cells to evaluate the uptake kinetics of EV subpopulations using fluorescence microscopy and flow cytometry.

RESULTS: Immunofluorescence images of EV donor cells showed clear endosomal staining pattern for CD63. In contrast, ITGA2 predominantly localized at the plasma membrane. Cluster analysis of dSTORM images showed low coincidence levels of CD63 and ITGA2 on single EVs. This was supported by EV immunoprecipitation followed by WB, revealing the majority of ITGA2 protein was not pulled down with CD63-positive EVs and v.v.. HaloTag fusion constructs of CD63 and ITGA2 allowed assessment of uptake kinetics of EV subpopulations individually.

CONCLUSIONS: CD63-positive and ITGA2-positive EVs represent distinct EV subpopulations. These subpopulations likely originate from different cellular localizations in the EV donor cells. Tracing these individual EV subpopulations using HaloTag fusion proteins and bright fluorophores, may be employed to understand differences in their intracellular trafficking and processing mechanisms.

Selected Oral Presentations

NLSEV2023-O12 Steven Wang

EV RNA profiling and deconvolution for outcome prediction in aggressive B-Cell lymphoma

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INTRODUCTION: Accurate risk stratification is essential for risk-adapted treatment strategies in Diffuse Large B-Cell Lymphoma (DLBCL). Extracellular vesicles (EVs) encapsulate a subtranscriptome that mirrors their cell of origin, thereby offering a glimpse of the immune system status and have biomarker potential. Here, we performed EV-RNA sequencing and deconvolution to elucidate EV origin and evaluate their prognostic utility.

METHODS: We sequenced 64 samples from the HOVON-152 trial (NCT03620578), a multicenter phase II single-arm interventional trial for High-Grade B-Cell Lymphoma (HGBL). EV-RNA sequencing was conducted following one treatment cycle. Responders (R) were identified by a complete metabolic response (CMR, Deauville score 1-3) on End-of-Treatment (EOT) imaging, while non-CMR responses were categorized as non-responders. SEC isolated EVs were confirmed with transmission electron microscopy (TEM), tunable resistive pulse sensing (TRPS), and western blot (WB). Library preparation was done with SMARTer and sequenced on the NovaSeq 6000 (Illumina). Subsequent analyses encompassed gene ontology (GO), gene set enrichment analysis (GSEA), and deconvolution using CIBERSORT. Then we developed and cross-validated an EV-messenger RNA (EV-mRNA) signature for EOT response prediction.

RESULTS: TEM after one cycle of R-CHOP revealed abundant particles < 200 nm and TRPS measured significantly higher particle concentration in EV-enriched fractions. pEVs were positive for CD63, CD81, flotillin-1, syntenin, HSP70, and negative for calnexin in accordance with the MISEV Criteria. GO highlighted a dominance of blood and immune cell-related genes. GSEA indicated upregulation of pathways tied to cell proliferation and cancer progression in non-responders (MYC Targets V1, PI3K/AKT/mTOR, IL-6/JAK2/STAT3 signaling pathway). CIBERSORT deconvolution pinpointed B-, T-, and myeloid cells as predominant sources of EV. An EV-mRNA Elastic Net signature, comprising six mRNAs, could predict EOT response, achieving a cross-validated AUC of 0.70.

CONCLUSIONS: EV-RNA profiling reveals immune system status in DLBCL patients after treatment initiation, providing a signal of tumor-immune interaction beyond conventional tumor-derived signals. An EV-mRNA signature predicted EOT response after one cycle of treatment in HGBL patients. Upon wider validation in DLBCL patients, plasma EV-RNA holds promise as a tool to assist risk-adapted treatment strategies in aggressive lymphomas.

Selected Oral Presentations

NLSEV2023-O13 Bárbara Adem

CD63 positive extracellular vesicles define a plastic network of communication in pancreatic cancer

Bárbara Adem [1,2], Nuno Bastos [1,2], Carolina F. Ruivo [1], Patrícia F. Vieira [1,3], Sara Sousa-Alves [1], Carolina Dias [1,3], Inês A. Batista [1,2], Dieter Saur [4,5], José C. Machado [1,6,7], Dawen Cai [8-10], Sonia A. Melo [1,6,7]
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INTRODUCTION: Pancreatic ductal adenocarcinoma (PDAC) is a deadly disease with very limited treatment options, making it essential to comprehend its biology to improve patient care. Extracellular vesicles (EVs) are known mediators of disease development, yet their behavior in living systems remains poorly understood. We aimed to map the spatiotemporal distribution of EVs in both healthy pancreas and PDAC to unravel their biological significance.

METHODS: We developed a CD63 multireporter mouse model (ExoBow) to trace spontaneous EVs communication. Using transgenic mice that faithfully recapitulate PDAC pathogenesis, we mapped the network of communication mediated by pancreatic cancer EVs within its microenvironment, and systemically, with other organs. Furthermore, we assessed the impact of inhibiting EVs secretion within the tumor microenvironment through the use of a PDAC conditional knock-out of Rab27a.

RESULTS: In the PDAC microenvironment, cancer cells establish preferential communication routes with cancer-associated fibroblasts and endothelial cells. The latter event is conserved in the healthy pancreas. Inhibiting EVs secretion in both scenarios significantly enhanced angiogenesis, underscoring the contribution of EVs to the vascularization in a healthy setting and the cancer context. Inter-organ communication is significantly increased in PDAC, being the kidneys and lungs the main targets. In a healthy setting, EVs accumulate preferentially in the bone-marrow, brain, and intestines. Interestingly, the thymus is a common route of communication in both settings.

CONCLUSION: In summary, our findings reveal that EVs orchestrate a well-organized communication network in vivo exerting local control over angiogenesis in both healthy pancreas and PDAC. Additionally, EVs consistently target the thymus in both contexts, suggesting a potential role in central immune surveillance and anti-tumor immune responses.

Selected Oral Presentations

NLSEV2023-O14 Crescenzo Massaro

Tumor-secreted EVs impair bone cancer therapy response by shaping the bone marrow immune environment

Crescenzo Massaro [1], Hilal Nur Sensoy [1], Manon Mulders [1], Celine De Schrijver [1], Cristina Gomez Martin [1], Juan Simon Nieto [1], Tonny Lagerweij [1], Alisha Atmopawiro [1], Jennifer Perez Boza [1], Maarten Bebelman [1], Leontien Bosch [1], Jan R.T. van Weering [3], Carmela Dell'Aversana [2,4], Lucia Altucci [2,4,5], Niels van de Donk [6], Cristina Giorgio [7], Laura Brandolini [7], Marcello Allegretti [7], Michiel Pegtel [1], S. Rubina Baglio [1]
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INTRODUCTION: The bone marrow is a supportive environment for cancer cells protecting them from the most advanced treatments. Recent observations suggest a critical role for functionally unique ''inflammatory'' mesenchymal stem cells (iMSCs) residing in the bone marrow stroma, yet their origin and function remain largely unknown. In this study we set out to study the direct and indirect (via iMSCs) effects of cancer extracellular vesicles (EVs) in tumor-promoting immune modulation, to identify combination therapies that can counteract stroma-induced drug resistance.

METHODS: Tumor EV-induced alterations of the MSC transcriptome were analyzed by RNA-seq and compared with scRNA-seq data of patient-derived iMSCs. Functional experiments with signaling receptor inhibitors were conducted to identify the mechanisms by which tumor EVs trigger iMSC development. Clinically available drugs against identified targets were evaluated in orthotopic xenograft mouse models of osteosarcoma. Tumor-associated bone marrow immune alterations were assessed in immunocompetent mouse models by spectral-flow cytometry-based deep immune profiling.

RESULTS: EVs from aggressive cancer cells induce an inflammatory MSC phenotype that recapitulates the single-cell transcriptome of iMSCs in bone cancer patient biopsies. Administration of iMSCs secreting high levels of IL6 and IL8 in osteosarcoma-bearing mice promotes lung metastasis formation and counteracts the efficacy of anti-metastatic drugs. Simultaneous blockade of IL8 and IL6 receptors with ladarixin and tocilizumab overcomes iMSC-induced drug resistance in vivo. Deep immune profiling revealed profound alterations of the bone marrow adaptive and innate immune compartments, which are in line with the increased levels of IL6 and IL8. Knockdown of EV biogenesis components determined reduced EV secretion in bone cancer cells enabling studies into the role of EVs in tumor-induced immune suppression.

CONCLUSION: Our observations establish cancer EVs as physiological triggers of tumor-promoting iMSCs, and iMSCs as major contributors to drug resistance in bone cancer. Future efforts will be directed towards investigating how tumor EVs contribute to the bone marrow immune alterations observed in immunocompetent mice.

Selected Oral Presentations

NLSEV2023-O15 Britta Bettin

Global inter-laboratory comparison study to standardize EV concentration measurements between 39 flow cytometers

Britta Bettin [1,2,3], Edwin van der Pol [1,2,3], Rienk Nieuwland [1,2]

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INTRODUCTION: Extracellular vesicles (EVs) in body fluids are potential disease biomarkers. To measure EV concentrations, most laboratories use flow cytometers (FCMs), but concentrations are incomparable between FCMs. To improve comparability, the METVES II consortium developed reference materials and methods to calibrate FCMs. This developed infrastructure was tested in a global inter-laboratory comparison study including 39 FCMs from 24 different laboratories.

METHODS: Concentrations of erythrocyte-derived (CD235a-PE) and platelet-derived (CD61 -APC) EVs were measured in stable and pre-labeled human plasma EV test samples (PEVTES). Flow rate was calibrated using solid silica beads, light scattering was calibrated using Rosetta calibration beads, and fluorescence was calibrated using MESF beads. EV concentrations were compared between FCMs within an EV size range of 242 – 1,000 nm and a fluorescence intensity >220 APC MESF, and >50 PE MESF.

RESULTS: Preliminary results from 15 FCMs show that FCM calibration is successful. For the platelet EV concentration, the coefficient of variation decreased from 70% without calibration to 27% after calibration.

CONCLUSION: This is the first inter-laboratory comparison study demonstrating that full flow cytometer calibration improves the comparability of EV concentration measurements between FCMs, thereby paving the road to clinically relevant multi-center biomarker studies on EVs.

Selected Oral Presentations

NLSEV2023-O16 Sajitha Sasidharan

Mechanical characterization of extracellular esicles using AFM-based nanoindentation methods

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INTRODUCTION: In the context of cellular evolution, extracellular vesicles (EVs) are conserved among all the three domains of life and it is believed EVs influence the process of cellular adaptation. Also, with mechanocues in cells influencing and regulating cellular behaviour, finding the impact of vesicle mechanics in shaping cellular evolution and representing the cellular microenvironment is a fascinating research question. The question about mechanocues is approached through an Atomic Force Microscopy(AFM)-based imaging and nanoindentation technique. However, the bottleneck in determining the vesicle mechanics through AFM is a well-defined experimental approach and model accounting for the fluidity of the vesicular membrane. Using a model based on adapted Canham-Helfrich theory, (i) the mechanics of vesicles and (ii) the extendibility of the model for EVs representing different microenvironments and different lipid packing is being explored.

METHODS: An AFM-based nanoindentation approach, based on an adapted Canham-Helfrich theory, was utilized to probe the distribution, morphology and biomechanical properties of the vesicles simultaneously.

RESULTS: The physical properties were evaluated of the vesicles grown under different conditions. The change in the microenvironment profoundly influenced the rate of release and the deformation of vesicles upon substrate adhesion. The influence of external conditions in EVs isolated from harsh environmental conditions and with a different lipid packing is under study. The obtained results and ongoing studies can improve the current understanding and further the knowledge relating the physical properties of EVs to their biological functions.

CONCLUSIONS: The advancement in understanding the mechanics influenced by the change in microenvironments will help identify the pathological state-dependent signature and design therapeutic options based on the knowledge derived from mechanobiology. The change in the mechanical response of the EVs isolated from organisms living in harsh environments will shed light on the evolution of cellular communication from the biophysics perspective. Currently, we are widening our knowledge of the adaptability of the theoretical model to establish a biomechanical landscape of EVs from all three domains of life.

Selected Oral Presentations

NLSEV2023-O17 Mendel Engelaer

A new practical procedure to set and report the trigger threshold of flow cytometers

Mendel Engelaer* [1,2], Mona Shahsavari* [1,2], Ton G. van Leeuwen [1,2], Edwin van der Pol [1,2]
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INTRODUCTION: Flow cytometry is a widely used method for quantifying extracellular vesicles (EVs). However, only EVs with signals exceeding the trigger threshold are counted. Consequently, the measured concentration of EVs depends on the trigger threshold, which differs among flow cytometers. For example, flow cytometers with a trigger threshold at 70 nm and 250 nm measure a 100-fold difference in EV concentration in urine. Two main problems of EV flow cytometry are (1) setting the trigger threshold relative to the lower limit of detection (LoD), and (2) reporting the trigger threshold in standard units. Therefore, we present a new practical procedure to measure the LoD, set the trigger threshold accordingly, and report them in standard units.

METHODS: To determine the LoD of the side scatter detector in a flow cytometer (Cytek Northern Lights), we measured the background noise by running MilliQ water while triggering on the edge of the background noise of a fluorescence detector. We defined the LoD as three standard deviations above the median background noise and propose to set the trigger threshold accordingly. To validate, we measured a mixture of polystyrene beads with a flat size distribution (30-100 nm) while setting the threshold below, at, and above the LoD. With a threshold below the LoD, the measured size distribution is expected to raise below the LoD due to the inclusion of background noise.

RESULTS: The median background noise, expressed as the optical diameter for polystyrene, was measured at 37 nm with a 6 nm standard deviation, leading to an LoD of 55 nm. When measuring the bead mixture with the threshold at 40 nm, the size distribution raised below 55 nm, as expected. Setting the threshold at 55 nm and 70 nm resulted in flat size distributions, confirming the proposed definition of the LoD and trigger threshold are sound. An optical diameter of 55 nm for polystyrene corresponds to an optical diameter of EVs of 91 nm.

CONCLUSION: We present a practical procedure to determine and report the LoD and set the trigger threshold accordingly. The procedure can be easily adopted by all flow cytometry users.

Sponsored Presentations

NLSEV2023-S1 Beckman Coulter

BEC LS Solutions for EV research, an update

Presenter: Daniel Lueert

(no abstract)

Sponsored Presentations

NLSEV2023-S2 IZON

Increasing small particle recovery and extracellular vesicle (EV) isolate purity with size exclusion chromatography

Presenter: Stephane Mazlan

AIMS: To develop a new series of size exclusion chromatography (SEC) columns for isolating smaller particles and compare extracellular vesicle (EV) isolate purity between our outgoing Legacy columns and our new Gen 2 columns.

METHODS: Working with ABT, we developed and tested qEV columns using a new 20 nm SEC resin aimed at improving isolation of smaller particles. Isolation efficiency was tested using carboxylated polystyrene particles of defined sizes. Elution profiles of EVs and proteins were determined using the Exoid and protein assays respectively. Additional lipoprotein analysis was conducted using ELISA.

RESULTS: In terms of protein contamination, EV isolates from Gen 2 columns were purer than their Legacy counterparts (35 nm Series: 3.73-fold purer, $p<0.01$; 70 nm Series: 6.38-fold purer, $p<0.05$). Additionally, Gen 2 columns showed an improved ability to remove lipoproteins (35 nm Series: 3.38-fold lower, 5.8% of input ApoB, $p<0.01$; 70 nm Series: 3.19-fold lower, 1% of input ApoB, $p<0.05$). ApoA retention was <0.0001% for both series of Gen 2 columns. Finally, the new 20 nm columns isolated significantly more smaller particles than 35 nm columns ($p<0.05$). We also observed a peak of protein consistent with exomeres and supermeres.

CONCLUSIONS: Gen 2 columns isolate purer EVs than did Legacy columns both in terms of protein and lipoprotein contamination. Our new qEV / 20 nm Series of columns isolate smaller particles, potentially including exomeres and supermeres.

Sponsored Presentations

NLSEV2023-S3 DENSsolutions

Controlling the biochemistry on-the-fly and visualizing reaction dynamics during in-situ Liquid Phase TEM: A strong tool for biopharmaceutical development

Presenter: Evgeniya Pechnikova

Recent developments in the pharmaceutical industry have involved a combinatorial approach. Resolving the details with (sub-)nanometer resolution has become a necessary requirement, as well as the need to visualize and understand the dynamics of the (bio)reactions that govern the different processes. Cryo Electron Microscopy has become a rapidly growing technique in the pharmaceutical industry due to its unique capability to uncover the complex interactions between e.g. drug and receptor, providing a powerful platform to accelerate drug discovery. However, the technique still suffers from intrinsic problems such as the liquid-air interface and not having the sample in its native liquid environment, making dynamic analysis difficult. As a consequence, not only the systematic errors are introduced, but the process prevents the possibility of enabling high-throughput studies. It is of utmost importance for the research community to find a strong complementary technique that can address these limitations of cryo-EM, to ensure the outcome of the research becomes stronger. In that sense, liquid phase transmission electron microscopy (LPTEM) has emerged as a powerful tool capable of providing unprecedented insights into the dynamics of various processes.

Sponsored Presentations

NLSEV2023-S4 Particle Metrix

NTA with ZetaView - More than just size and concentration

Presenter: Ingrid Bloss

(no abstract)

Poster Presentations

NLSEV2023-P1: Joel E.J. Beaumont

GABARAPL1 is essential in extracellular vesicle cargo loading and metastasis development

Joel E.J. Beaumont [1], Jinzhe Ju [1], Lydie M.O. Barbeau [1], Imke Demers [1,2], Kim G. Savelkouls [1], Kasper Derks [3], Freek G. Bouwman [4], Marca H.M. Wauben [5], Marijke I. Zonneveld [1], Tom G.H. Keulers [1], Kasper M.A. Rouschop [1]

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INTRODUCTION: Hypoxia is a common feature of tumours associated with poor prognosis due to increased resistance to radio- and chemotherapy and enhanced metastasis development. Extracellular vesicles (EV) contribute to metastasis development and previously we demonstrated that GABARAPL1 is required for EV secretion during hypoxia. Here, we explored the role of GABARAPL1+ EV in the metastatic cascade.

METHODS: GABARAPL1 deficient and control MDA-MB-231 cells were injected in murine mammary fat pads. Lungs were dissected and analysed for human cytokeratin 18. EV from control or GABARAPL1 deficient cells exposed to normoxia (21% O2) or hypoxia (O2 <0.02%) were isolated and analysed using immunoblot, nanoparticle tracking analysis, high resolution flow cytometry, mass spectrometry and next-generation sequencing. Cellular migration and invasion were analysed using scratch assays and transwell-invasion assays, respectively.

RESULTS: The number of pulmonary metastases from GABARAPL1 deficient tumours decreased by 84%. GABARAPL1 deficient cells migrate slower but display comparable invasive capacity. Both normoxic and hypoxic EV contain proteins and miRNAs associated with metastasis development. In line, these EV increase cancer cell invasiveness. Although GABARAPL1 deficiency alters EV content, it does not alter the EV-induced increase in cancer cell invasiveness.

CONCLUSIONS: GABARAPL1 is essential for metastasis development. This is unrelated to changes in migration and invasion and suggest that GABARAPL1 or GABARAPL1+ EV are essential in other processes related to the metastatic cascade.

Poster Presentations

NLSEV2023-P2: J.C. van Maanen

The effect of notochordal cell-derived extracellular vesicles on degenerated intervertebral disc tissue

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BACKGROUND: Intervertebral disc (IVD) degeneration is related to chronic low back pain, causing a considerable societal burden. The degenerative process is characterized by matrix breakdown, exacerbated by pro-inflammatory cytokines. Extracellular vesicles (EVs) of notochordal cells (NCs), juvenile disc cells in healthy discs, are considered a promising regenerative approach. Thus far, their effects have only been studied in cell culture. For clinical translation, it is essential to demonstrate their effects in conditions that resemble the in vivo degenerative environment. Therefore, this study evaluated the biological effects of NC-EVs on patient-derived IVD tissue explants.

METHODS: EVs were isolated from conditioned media from pig NC-rich tissue (pNC) via differential centrifugation and size exclusion chromatography and characterized (nanoparticle tracking analysis and protein marker identification). IVD tissue explants were obtained from four patients with moderate disc degeneration undergoing standard-of-care surgical treatment (ethical approval: 09/H1308/70). The IVD tissue was cultured for two weeks in conditions mimicking the degenerated disc environment (with or without interleukin 1β (IL-1β)) in the presence or absence of pNC-EVs. The pNC-EV-associated anti-catabolic and immunomodulatory effects were evaluated and compared to pig donor-matched EV-depleted control medium.

RESULTS: The 2-week treatment of human explants with NC-EVs did not induce statistically significant effects on DNA content or matrix composition of the tissue explants. NC-EV treatment decreased the secretion of chemokines CCL2 (68%) and CXCL1 (100%), while the cytokine IL-6 was increased (100%) in the culture media when compared to control media. NC-EVs decreased immunopositivity of IL-1β (66%) and its receptor IL-1R1 (66%). The NC-EV-associated immunomodulatory effects were only observed in the presence of IL-1β in the culture. These effects were absent in explants treated with EV-depleted media.

CONCLUSIONS: This is the first study showing the effects of NC-EVs in a clinically relevant ex vivo model for disc degeneration. Initial results indicate that NC-EVs may reduce the effects of interleukin 1β in the degenerating disc, which could modulate the degenerative cascade. In a parallel study, mass spectrometry is ongoing on pNC-EVs to elucidate their potential mechanism of action and therapeutic potential in intervertebral disc-related low back pain.

Funded by European Union's Horizon 2020 program iPSpine (No.825925).

Poster Presentations

NLSEV2023-P3: Jillian W.P. Bracht

A novel spectral flow cytometry workflow to assess the RNA topology of submicron particles

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INTRODUCTION: Extracellular vesicles (EVs) carry different types of cargo, including RNA, providing candidate biomarkers for disease. However, there is no consensus or experimental proof on the exact topology (location) of EV-RNA. This knowledge gap leads to irreproducible results and hampers standardization in EV-RNA research. Within the RNA-top project we aim to decipher if EV-RNA is located on the surface of EVs, inside EVs, or both. Currently, there are no workflows to explore single EV-RNA topology and no model systems to validate such workflows. Here, using a liposome model system, we developed a spectral flow cytometry (sFCM)-based workflow to detect RNA on the membrane surface.

METHODS: Positively charged- and negatively charged liposomes (100 nm, DOTAP:HSPC:Chol=1:4:3 and DSPG:HSPC:Chol=1:4:3) were prepared using a hydration, freeze-thaw and extrusion method. Total RNA (negatively charged) was isolated from a BxPC3 cell line, and bound to the membrane surface of the liposomes based on electrostatic interactions. A fluorescent RNA dye (SYTO) was used to detect the RNA. The concentration of liposomes and RNA-bound liposomes was measured using calibrated sFCM (Cytek Northern Lights, diameter >145 nm).

RESULTS: The total concentration of positively charged- and negatively charged liposomes was 1.9E13 and 2.4E13 liposomes/mL (based on side scatter), respectively. The concentration of liposomes containing fluorescent RNA on the membrane surface was 3.4-fold higher for the positively charged liposomes, compared to the negatively charged liposomes or the control sample (RNA in water). These results indicate that unbound RNA is below the detection range of the instrument, and can only be detected by sFCM upon electrostatic binding to the liposomes.

CONCLUSION: We have developed a workflow to detect fluorescent RNA on the membrane surface of a liposome model system using sFCM. Further research will focus on optimization of the presented workflow, development of workflows to detect intra-vesicular RNA, and extrapolation of these workflows to EV samples.

Poster Presentations

NLSEV2023-P4: Onno J. Arntz

Protein cargo of plasma-derived extracellular vesicle reflect different organ involvement in systemic sclerosis patients

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BACKGROUND: Systemic sclerosis (SSc) is an autoimmune disease characterized by vasculopathy and tissue fibrosis of the skin and internal organs. Approximately 12% of SSc patients will develop pulmonary arterial hypertension (PAH), a leading cause of death in these individuals. The pathophysiology of SSc is complex and not completely understood, but it is speculated that extracellular vesicles (EVs) play a role. EVs are cell-derived membrane vesicles, and their content reflex that of the donor cells.

OBJECTIVE: This study aimed to provide insight into ongoing disease processes in SSc patients by studying the protein cargo of circulating EVs.

METHODS: Protein levels of plasma EVs (pEVs) from 20 SSc patients and 10 age- and sex-matched healthy donors (HC) were determined using mass spectrophotometry, and were correlated to laboratory and clinical parameters. Additionally, the positively correlated pEV proteins were tracked back to their original source/organ, using protein atlas (proteinatlas.org).

RESULTS: Concentration, size, and protein content of pEVs were comparable between SSc patients (1.62*1010 particles/ml, 109nm, 3.20fg protein/particle) and HC (2.61*1010 particles/ml, 118nm, 2.36fg protein/particle). Proteomic analysis revealed 605 (>99%) overlapping proteins in SSc-pEVs, compared to HC-pEVs. Ten proteins showed significant enrichment, of which three proteins (ORFM1, C1R, APOC1) were positively correlated with uric acid levels, and one (DSC3) with the modified Rodnan skin score (mRSS). Uric acid levels may predict the development of PAH in systemic sclerosis (SSc). Interestingly, the pEV proteins that positively correlated with uric acid levels were mainly liver proteins, suggesting that the liver contributes to PAH development, which has been previously reported. Notably, the high number of unique SSc-pEV proteins correlated with DSC3 (41) predominantly originated from the skin, as determined using protein atlas. In combination to the previous observed correlation with mRSS, it shows that EVs released from affected skin contributes to pEVs.

CONCLUSIONS: By studying the protein cargo of SSc-pEVs, we can observe skin and liver involvement within SSc. This observation underscores the potential of analyzing circulating EVs to gain deeper insights into ongoing disease processes in patients.

Poster Presentations

NLSEV2023-P5: D. Corraini

A pro-degenerative environment promotes the regenerative potential of extracellular vesicles derived from notochordal cells

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INTRODUCTION: Extracellular vesicles (EVs) are considered a promising treatment for intervertebral disc (IVD) degeneration-related low back pain. EVs derived from porcine notochordal cells (NCs), juvenile inner disc cells, induce proliferation in both human and dog degenerate disc cells, the nucleus pulposus cells (NPCs) and limited healthy extracellular matrix production. We hypothesize that pro-degenerative stimuli induce NCs to produce more potent regenerative EVs, in the attempt to counterbalance the micro-environment stress.

METHODS: NC-rich explants were isolated from one porcine spine and cultured at 370C, 5% O2 for 4 days in low glucose (1 gr/L) DMEM, 7.1 pH, 450 mOsm medium (healthy, H-EVs), or 6.8 pH, 350 mOsm medium (pro-degenerate, D-EVs). Inflammation of treated explants was confirmed looking qualitatively at matrix deposition and NCs phenotype via (immuno)stainings. NC-EVs were isolated from the conditioned media via differential centrifugation, followed by Size Exclusion Chromatography. The particle size and NC-EVs concentration were determined via Nanoparticle Tracking Analysis. The regenerative potential of H-EV and D-EV, including their respective EV-depleted controls, was tested on NPC pellets using histological and biochemical assays.

RESULTS: Comparable average number and size of D-EVs (3*1010 particles/gram tissue; 199 nm) and H-EVs (2.8*1010 particles/gram tissue; 195 nm) was isolated from the conditioned media. Histological and biochemical analysis showed comparable GAG deposition and DNA levels in NPC pellets treated with D-EVs and H-EVs, and their respective EV-depleted controls. Nonetheless, the total GAG produced (pellets + medium) by NPCs treated with D-EVs was 2.8-fold higher compared to H-EVs. Furthermore, both H- and D-EVs induced higher total GAG production than the respective EV-depleted controls (14.5-fold and 3.4-fold, respectively).

CONCLUSIONS: This preliminary study showed an EV-specific positive effect on healthy matrix production by NPCs derived from degenerate discs. Despite comparable particle numbers, the beneficial effect was evident for NC-EVs derived from a pro-degenerative environment compared to a healthy environment. These data suggest that the difference between D-EV and H-EV anabolic effects on NPCs resides within the EV cargo, rather than the particle number. Therefore, follow up work focuses on replicating the work and expanding on proteomic and RNA-sequencing studies, aiming to decode the differential EVs content composition.

Poster Presentations

NLSEV2023-P6: Maarten P. Bebelman

Viral GPCR-activated EV release as a novel therapeutic target in brain cancer

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INTRODUCTION: US28 is a viral G-protein coupled receptor (vGPCR) encoded by the human cytomegalovirus (HCMV). A HCMV infection is often asymptomatic, however, in immune compromised individuals such as cancer patients it can give rise to complications. In glioblastoma, a fast-growing and aggressive form of brain cancer, it has been demonstrated that US28 expression can dysregulate oncogenic signaling pathways, resulting in sustained cell proliferation, immune evasion, and angiogenesis.

METHODS/RESULTS: We have found that US28 is secreted from GBM cells on small extracellular vesicles (EVs). Electron microscopy revealed that the vGPCR US28 is sorted into intraluminal vesicles of multivesicular bodies (MVBs). Consequently, these MVBs fuse with the plasma membrane to release the vesicles into the extracellular environment as was demonstrated through live-cell total internal reflection fluorescence (TIRF) microscopy. Here, US28 retains its chemokine scavenging function. A nanoBRET based approach illustrated that US28 positive EVs interfere with the CX3CL1-CX3CR1 signaling axis by binding its ligand CX3CL1. Our ongoing research aims to elucidate the mechanism that US28 exploits to be selectively sorted into and released via EVs. NanoBRET will allow us to probe known/suspected-interactors, whereas a proximity-ligation (TurboID) approach will be employed to identify novel components. In-house developed nanobodies (antibody fragments) will serve as research tools to modulate US28 function. We will determine the function of US28 containing EVs beyond its scavenging properties, including horizontal transfer of US28 to non-infected cancer cells, to understand the contribution of US28 containing EVs within the tumor microenvironment.

CONCLUSIONS: Altogether, our findings identify a novel viral-encoded GPCR as exosomal cargo, that may scavenge chemokines and aid in immune evasion of HCMV-infected cancer cells.

Poster Presentations

NLSEV2023-P7: Kelly Warmink

Mesenchymal Stem/Stromal Cells-derived extracellular vesicles as a potentially more beneficial therapeutic strategy than MSC-based treatment in a mild metabolic osteoarthritis model

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BACKGROUND: Mesenchymal stromal/stem cells (MSCs) and MSC-derived extracellular vesicles (MSC-EVs) hold promise as a disease modifying treatment in Osteoarthritis (OA). Obesity, and its associated inflammation, contribute to OA development and metabolic OA represents a specific and significant group of the OA patient population. Given their immunomodulatory properties, MSC and MSC-EVs are especially interesting for this group of patients as a therapeutic option. Here, we were the first to compare the therapeutic efficacy of MSCs and MSC-EVs in a mild OA model taking these metabolic aspects into consideration.

METHODS: Male Wistar-Han rats (Crl:WI(Han) (n=36) were fed a high fat diet for 24 weeks, with unilateral induction of OA by groove surgery after 12 weeks. Eight days after surgery rats were randomized in three treatment groups receiving MSCs, MSC-EVs or vehicle injection. Pain-associated behavior, joint degeneration, and local and systemic inflammation were measured.

RESULTS: We demonstrated that despite not having a significant therapeutic effect, MSC-EV treatment results in lower cartilage degeneration, less pain behaviour, osteophytosis and joint inflammation, than MSC treatment. Suggesting that MSC-EVs could be a more promising therapeutic strategy than MSCs in this mild metabolic OA model.

CONCLUSION: In summary, we find that MSC treatment has negative effects on the joint in metabolic mild OA. This is an essential finding for the significant group of patients with metabolic OA phenotype, and might help to understand why clinical translation of MSC treatment shows varying therapeutic efficacy thus far. Our results also suggest that MSC-EV-based treatment might be a promising option for these patients, however MSC-EV therapeutic efficacy will need improvement.

Poster Presentations

NLSEV2023-P8: Marie Burt

Bacterial outer membrane vesicles protect bacteria from polymyxins by lipid A

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INTRODUCTION: Klebsiella pneumoniae (Kp) is a pathogenic bacterium that can cause pneumonia. The emergence of hypervirulent and multidrug-resistant strains has made Kp one of the antibiotic-resistant pathogens prioritized by the WHO. It is essential to understand its mechanisms of pathogenicity. Our study investigates the role of bacterial outer membrane vesicles (OMVs), in the interplay between antibiotics and bacteria. Specifically, we analyze the impact of stress conditions on OMV release and the effect on antibiotic resistance in Kp.

METHODS: To investigate the impact of different antibiotics on vesiculation in Kp, Kp was subjected to antibiotic-induced stress. OMVs from both Polymyxin B (PB) treated and control Kp were isolated and characterized to examine their influence on antibiotic resistance in vitro, ex vivo and in vivo. Protein profile of Kp treated with different antibiotics and addition of OMVs was studied, along with the impact of PB treatment on the lipidome of Kp and their respective vesicles. Artificial vesicles were generated to explore how changes in the lipid composition affect antibiotic resistance.

RESULTS: Kp releases OMVs of varying size and amount under different stress conditions. Exposure to PB leads to significant changes in vesicle composition, not associated with an upregulation of PB resistance genes. Lipidomics analyses reveal that PB alters OMVs composition but not of the source bacteria. Importantly, OMVs protect Kp from PB in a dose-dependent manner and reduce antibiotic stress response confirmed by proteomics. OMVs also confer protection against PB in E. coli, Salmonella enterica, and Pseudomonas aeruginosa. We generated artificial vesicles that validate this. OMVs were also protective against the polymyxin colistin, but not against meropenem and gentamicin. Finally, OMVs protect Kp against PB in ex vivo and in vivo infection models.

CONCLUSION: Our study highlights the critical role of OMVs in promoting antibiotic resistance in Kp and other antibiotic-resistance priority pathogens. Our data suggest that OMVs might promote bacterial replication and subsequent spreading in the host when the infection is treated with polymyxins.

Poster Presentations

NLSEV2023-P9: Luuk van de Schepop

Bottom-up synthesis and purification of synthetic extracellular vesicle mimetics

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INTRODUCTION: Extracellular vesicles (EVs) are promising drug delivery systems due to their biological stability, intrinsic targeting properties, and ability to overcome natural barriers. However, limitations such as cumbersome production and isolation procedures, batch-to-batch variability, and challenges related to efficient cargo loading limit their potential for clinical applications. Here, we introduce EV mimetics, which are synthetic lipid vesicles with incorporated full-length membrane proteins. EV mimetics mimic functional characteristics of EVs, while retaining the scalability and controllability of synthetic systems. As a model EV protein, we used CD47, which serves as a “don’t eat me” signal to macrophages. We aimed to synthesize, purify, and characterize CD47 EV mimetic proteoliposomes.

METHODS: To prepare CD47 EV mimetics, CD47 was synthesized by in vitro transcription and translation (PURExpress) in the presence of liposomes, allowing incorporation of full-length membrane proteins into synthetic vesicles. After production, EV mimetics were isolated using sucrose density gradient ultracentrifugation and purified using a Twin-Strep-Tag incorporated in the CD47 construct. Characterization of EV mimetics was performed using western blot for protein content, fluorescent lipid quantification for liposome quantification, proteinase K protection assay for topology, and immuno-electron microscopy for visualization.

RESULTS: Firstly, successful production of CD47 EV mimetics was revealed by western blot, lipid quantification, and immuno-electron microscopy upon sucrose density gradient ultracentrifugation. Secondly, lipid concentration, lipid formulation, and plasmid concentration were optimized leading to an optimal yield of CD47 EV mimetics. Thirdly, we could demonstrate that EV mimetics prepared with CD47 fused to a Twin-Strep-Tag can be enriched from liposomes without incorporated CD47 by affinity chromatography. Finally, we demonstrated that CD47 is incorporated in the liposomes with the correct topology.

CONCLUSIONS: We demonstrate successful synthesis and purification of CD47 EV mimetics using a scalable procedure. This is an important step towards the development of synthetic drug delivery vehicles with enhanced EV-inspired functionality. Further research will be done to standardize this procedure for incorporation of other membrane proteins, and for incorporation of multiple membrane proteins simultaneously. Additionally, functionality of the membrane proteins will be assessed.

Poster Presentations

NLSEV2023-P10: M.N. Monteiro

Exosome pathway inhibition as therapeutic strategy in colorectal cancer

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INTRODUCTION: Tumor-derived exosomes play a pivotal role in driving cancer progression by shaping the tumor microenvironment. Targeting exosome secretion emerges as a promising avenue for therapeutic intervention. Understanding the key protein players orchestrating exosome biogenesis is essential for devising effective treatment strategies.

METHODS: We collected tumor and normal adjacent tissue secretome ex vivo from 22 patients with colorectal cancer (CRC). Mass spectrometry-based proteomics was performed on tissue and exosome fractions isolated using Vn96 peptide. Comparative analysis between CRCs and controls was performed with bb-test and biology mining was performed with Gene Set Enrichment Analysis (GSEA) and Gene Ontology. To identify novel drug targets, we conducted targeted data mining of the exosome biogenesis pathway and identified upstream kinases from kinase-substrate public databases. CRC cell lines were subjected to kinase inhibitors and secreted exosomes were quantified by EVQuant, and characterized using immunofluorescence staining and proteomics.

RESULTS: The multi-compartment CRC dataset comprised 3353 exosome proteins and unsupervised clustering completely separated tumor and normal samples. Biology mining revealed some shared cancer hallmark enrichment in tumor tissue and tumor exosomes, including down-regulation of apoptosis, and up-regulation of G2M checkpoint, gene expression, and protein folding. Additionally, apical junction and cell adhesion were regulated predominantly in exosomes, highlighting their potential as therapeutic targets. Targeted data mining to the exosome biogenesis pathway revealed 12 up-regulated proteins and 14 proteins with increased cellular phosphorylation levels. Upstream kinase inhibition led to a reduction in vesicle release in a cell-type and vesicle-type dependent manner, quantified in cell-conditioned media. Specifically, Casein Kinase 2 (CK2) inhibition notably decreased CD63+ vesicles. Immunofluorescence staining for the multivesicular body marker CD63 confirmed reduced intensity post CK2 inhibition. Proteomic analysis of exosomes derived from the HCT116 cell line revealed significant alterations including down-regulation of epithelial-mesenchymal transition and angiogenesis in vesicles released after CK2 inhibition.

CONCLUSION: Our study identifies potential clinically actionable drug targets within the exosome-release pathway of CRC. Importantly, we discovered that inhibiting CK2 activity exerts a discernible impact on tumor invasion, presenting a novel therapeutic avenue against CRC. These findings underscore the significance of targeting exosome biogenesis in the quest for effective cancer therapies.

Poster Presentations

NLSEV2023-P11: Hailun Chen

Enhancing the targeting ability of RNA therapeutics-loaded extracellular vesicles to pancreatic cancer by glycoengineering

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INTRODUCTION: In vivo studies have shown that extracellular vesicles (EV) from fibroblasts loaded with siRNAs can target pancreatic ductal adenocarconoma (PDAC), but with low specificity and efficiency (Katrekar et al.2017). Macropinocytosis may drive internalization of EVs in mutant KRAS cancer cells that could be exploited for drug delivery. Previously, we demonstrated that immune-cell EVs efficiently mediate RNA delivery for targeted gene repression (Pegtel et al., 2010). Glycosylation may enhance EV cell-specific targeting and cargo delivery as shown for many viruses.

Hypothesis: We hypothesized that the targeting ability and therapeutic efficiency of RNA-loaded EVs could be further improved by understanding the glycosylation status of the EV surface. We proposed that once EVs selectively bind to tumour cells they will be internalized through macropinocytosis and escape destructions. To test this hypothesis, we will characterize the mechanism of EV uptake by KRAS-mutant PDAC cancer cells.

METHODS: We designed a KRAS-mutant model to explore the role of active macropinocytosis and glycosylation in EV uptake . To this end, we first measured the uptake of 70 kDa FITC-dextran by cancer cells as a marker of macropinocytosis by fluorescence microscopy and FACS. EIPA is used as an inhibitor of macropinocytosis. Serum starvation are used to increase the level of macropinocytosis. Subsequently, we also measured the uptake of PKH67-stained EVs with different glycosylation status by PDAC cells.

RESULTS: Fluorescence microcopy shows that dextran enters mutant KRAS cells via macropinocytosis conforming previous reports. FACS showed that the uptake of FITC-dextran by recipient cells increased with incubation time, in both KRAS-mutant cancer cells and is enhanced under serum-starvation, a driver of macropinocytosis. Fluorescence microscopy and FACS results showed that PDAC EVs are taken up in a dose-dependent manner.

CONCLUSIONS: We developed a cell model the biochemical properties and dynamics of EV uptake by KRAS-mutant cancer cells. Screening strategies will be applied to enhance the drug delivery potential of EVs.

Poster Presentations

NLSEV2023-P12: Simone Foderaro

A 3D Bone Marrow Model to study the role of EV-mediated communication in bone metastasis formation

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BACKGROUND: Bone metastases are common in most tumors and determine increased risk of death and loss of quality of life. Little is known about the mechanisms that cancer cells utilize to convert the rich immunological landscape of the bone marrow in a tumor-supportive one. Extracellular vesicles (EVs) proved to play a major role in many if not all steps of cancer progression. We generated a robust and easily reproducible immune 3D model mimicking the macro- and microstructure of the bone marrow. Population of the model with engineered tumor cells enables studies into the role of extracellular vesicles (EVs) in tumor-induced immune suppression.

METHODS: The 3D bone marrow (BM) system was assembled by combining a commercially available collagen-gelatine sponge with a plasma-derived hydrogel. Immune cells were obtained from peripheral blood of healthy donors or murine bone marrow. We stably expressed shRNAs against EV biogenesis components (Rab11b, Rab35 and syntenin) in human and murine tumor cells (MDA-MB-231, 4T1). The impact of the knockdowns on EVs secretion was assessed by a NanoLuc-CD63 assay. Skewing of immune cells was assessed by spectral flow cytometry-based high dimensional immune profiling.

RESULTS: Our 3D BM multicomponent system successfully retained cancer and immune cells, allowing intercellular interactions in experimental time frames. NanoLuc-CD63 assay showed a reduction of CD63-positive EVs secretion after constitutive knockdown of Rab11b, Rab35 and Syntenin in human and murine tumor cells. Deep immune profiling revealed immune alterations in tumor-bearing vs control 3D BM systems, which differed from those observed in conventional 2D cultures.

CONCLUSIONS: We successfully developed a 3D bone marrow model that supports cell viability and allows cell-cell interaction for the study of tumor-induced immune modifications. Preliminary results support a role for EVs in bone marrow immune suppression that warrants further investigation.

Poster Presentations

NLSEV2023-P13: Misko Bobeldijk

Converging pathways in exosome secretion

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INTRODUCTION: Exosomes, small vesicles facilitating cellular component exchange, are vital for maintaining homeostasis but undergo alterations in various diseases. These changes significantly impact disease progression in conditions such as diabetes, Parkinson's disease, and cancer. Targeted inhibition of exosome secretion holds promise for improving prognoses. To date, a broad spectrum of Rab GTPases have been implicated in different exosome secretory pathways, including Rab27a/b and Rab11. Yet, much remains unclear about the exact mechanism(s) involved in exosome secretion and to what extent the different pathways might overlap or compensate for each other. Here, we shed light upon the involvement of Munc13-4, a shared effector of Rab27 and Rab11, in exosome secretion. Furthermore, we implicate a novel small Rab GTPase, Rab37, in CD63+-exosome secretion.

METHODS: To study the effect of Munc13-4 and Rab37 on CD63+-compartments, we used mutational analysis and small-molecule targeting in HeLa wild-type cells. We visualized this in fixed cells and employed live-cell dual-color total internal resonance fluorescence (TIRF) microscopy to visualize CD63+-multivesicular bodies (MVBs) fusing with the plasma membrane (PM) in single cells, combined with conventional biochemical analysis.

RESULTS: Munc13-4 was found to be absent from the MVB/PM fusion site but played a crucial role upstream in MVB/PM fusion. Rab37, on the other hand, was implicated in the late stages of exosome secretion. Overexpression of Rab37 enhanced MVB/PM fusion, exhibiting kinetics distinct from Rab27a. Notably, Rab37's stimulatory effect was not dependent on its GTP/GDP state but rather on phosphorylation. Knockout of Rab37 had a significant impact on MVB/PM fusion dynamics.

CONCLUSIONS: Inhibiting Munc13-4 function severely impacted exosome secretion, highlighting its essential role. Munc13-4 serves as a shared effector for Rabs 11 and -27, which were previously associated with exosome secretion, as well as Rab37. Rab37 emerges as a novel regulator of exosome secretion, distinct from Rab27a. Interestingly, Rab37's function in exosome secretion is independent of GTP/GDP state but is instead regulated by phosphorylation at the T172 residue. Ongoing investigations suggest Rab37's necessity in amphisome formation and, ultimately, exosome release through secretory autophagy.

Poster Presentations

NLSEV2023-P14: Hilal Nur Sensoy

Rational design of EV-mediated CRISPR-Cas9 delivery for cancer gene therapy

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INTRODUCTION: The advent of CRISPR-Cas systems opened exciting opportunities for therapeutic gene editing. However, delivering CRISPR components safely and effectively into target cells in vivo remains an unmet challenge. Several delivery methods have been proposed, but they present significant drawbacks, such as immunogenicity, unfavourable biodistribution and rapid clearance. To tackle this hurdle, our research focused on Extracellular Vesicles (EVs) as natural, non-immunogenic communication devices that can horizontally transfer functional RNA molecules between cells. Based on our knowledge on the mechanisms underlying cell-mediated RNA packaging into EVs, we hypothesized that sorting of the CRISPR guide RNAs (gRNAs) into EVs can be enhanced by rational design of their 3'-end.

METHODS: We designed a library of p53-targeting gRNA variants with different 3'-ends and tested their functionality and their accumulation into EVs. gRNA functionality was assessed by transducing RPE cells with the p53 gRNA variants followed by clonogenic assay under Nutlin-3a conditions. gRNA levels in cells (MDA-MB-231 and 143B) and EV fractions were determined via qPCR.

RESULTS: Based on the Nutlin-3a assay, all gRNA variants are at least as functional as the conventional (unmodified) gRNA. In addition, we found that some 3' U-richvariants are more efficiently sorted into EVs as compared to their conventional counterpart in two independent cell systems.

CONCLUSIONS: Our data show that engineering the gRNA 3'-end can enhance sorting into EVs. Future efforts will aim at addressing whether the EV-associated gRNA variants can be functionally transferred into recipient cells in vitro.

Poster Presentations

NLSEV2023-P15: Agustin Enciso-Martinez

Developing a particle atlas for biomarker exploration through cryo-electron microscopy and artificial intelligence

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INTRODUCTION: A pre-requisite for EV biomarker research is to know the sub-µm particle profile in body fluids of healthy individuals. Current methods are unable to capture the full size range of EVs and other sub-µm particles, due to the lack of sensitivity to detect the smallest yet probably most abundant EVs. As such, the majority of EVs, including those uniquely present in patient body fluids, remains virtually unexplored. To date, the only technique that is able to directly visualize the broad spectrum of EVs, which can be as small than 30 nm, is cryo-electron microscopy (cryo-EM). Cryo-EM clearly shows the lipid bilayer of single EVs, and hence is capable of label-free identification of EVs. Here, we developed a cryo-EM EV imaging set-up that enables the creation of a particle atlas for biomarker exploration through cryo-electron microscopy and artificial intelligence.

METHODS: To gain fundamental insight into the sub-micrometer particle profile of EVs, we established a cryo-EM EV imaging set-up, which includes sample purification, sample preparation, data collection and image analysis. To account for the low concentration of EVs in body fluids and conditioned medium, hundreds of images were collected using an automated data collection scheme. EV segmentation was performed using convolutional neural networks.

RESULTS: EVs were rapidly localized using artificial intelligence, creating a unique image library of all EVs and other particles that are present in samples, such as plasma. To identify specific subsets of EVs, we are currently correlating cryo-EM images with fluorescence microscopy in a cryo-correlative light and electron microscopy (cryo-CLEM) workflow, enabling the imaging of the same particles by both methods. Additionally, by cryo-electron tomography (cryo-ET) we explored the 3D structure of single EVs, and identified protein/glycan structures present on the EV surface and possible internal contents, such as DNA and RNA.

CONCLUSION: In conclusion, this platform and atlas will advance our understanding of the particle profile of human body fluids, and this knowledge will be indispensable to further optimize detection and isolation methods of EVs for biomarker exploration.

Poster Presentations

NLSEV2023-P16: Ardalan Mansouri

Generating prostate cancer cell subpopulations with low and high levels of uptake of extracellular vesicles

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INTRODUCTION: Prostate cancer cells communicate through extracellular vesicles (EVs), yet they exhibit significant variability in EV uptake, ranging from none to tens of EVs per cell. This raises questions about the distinctiveness of these cell subpopulations and the feasibility of selecting subclones with various EV uptake tendencies. In this study, we repeatedly sorted cells with low and high EV-uptake to study their cellular and genetical differences.

METHODS: DU145 prostate cancer cells were exposed to PKH26-labeled EVs for 3 hours. We employed fluorescence-activated cell sorting (BD FACSAria IIu) to segregate cells into the lowest and highest 10% based on the PKH26 median signal. These low and high EV uptake subpopulations were cultured separately, with sorting conducted approximately every 10 days for lowest or highest EV uptake cells. The FACS was performed for six rounds within a 50-day timeframe, and the results were confirmed by microscopy. Subsequently, the cells were cultured without sorting, with sporadic uptake signal assessments. We evaluated different cell populations for proliferation using microscopy-based cell counting, and cell pellets were stored for RNA analysis.

RESULTS: Through six rounds of sorting, the low- and high-uptake DU145 cell populations exhibited substantial divergence. The median PKH26 FACS signal ratio between high- and low-uptake cells increased from 1.5 to 4, 4.8, 13 (two rounds), and 14.5. Following the sixth sorting round, by just culturing without sorting, we assessed the stability of the subpopulations. A month later, due to continuous enhancement in EV uptake by the high-uptake subpopulation, the median high to low subpopulation uptake ratio surged from 14.5 to 30. FACS analysis demonstrated an enlargement of the high-uptake subpopulation cells, that could have contributed to the elevated EV uptake. Proliferation assays unveiled a slightly (1.2x) increased growth rate in the high-uptake population compared to the low-uptake subclone and unsorted DU145 cells. RNA sequencing analysis will soon explore pathway changes related to EV uptake.

CONCLUSIONS: We successfully generated distinct subpopulations from a cell line characterized by either low or high EV uptake through serial sorting. Further investigation is required to elucidate molecular differences and assess the stability of these subclones during continuous passaging.

Poster Presentations

NLSEV2023-P17: M. Laçin

Extracellular vesicles for expression of kinase inhibiting peptides to eradicate glioblastoma cells

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INTRODUCTION: Glioblastoma is the most aggressive and malignant type of primary brain cancer with a dismal prognosis of just 10 to 13 months following diagnosis. Targeting protein kinases (“kinome targeting”) may potentially normalize glioblastoma cells and is considered to be an innovative way to provide precision treatment to cancer patients. One promising approach for targeting kinases is the use of kinase inhibiting peptides (KIPs). In this study, we intend to develop therapeutic extracellular vesicles (EVs) that are loaded with DNA to express KIPs as a new type of cancer treatment.

Method: As a first step we used myristoylated KIPS (Myr-KIPs), which consist of short peptide sequences with a myristoylation fatty acid chain attached to their N-terminal side, as a way to screen the efficacy of KIPs. The objective was to develop proof-of-concept data demonstrating the effectiveness of different KIPs in attenuating cell viability of glioblastoma cell lines. We included Myr-KIPs targeting DNA-PK, CDK1/2 and PKC.

RESULTS: Varying effects of the KIPs were observed and in particular one of the KIPs against CDK1/2 demonstrated a profound anti-tumour effect (decrease of approximately 90% in cell viability on U87 and GS.607 cells after incubation with 80µM Myr-KIP). We also performed mass-spectrometry-based proteomics analysis to gain more insight into the mode-of-action of the KIPs, which provided evidence on intended effects on the targeted kinase pathway. Supported by these findings we next created EVs loaded with full-length DNA of an Adenoviral vector (Adenosomes) encoding sequences of KIPs targeting CDK1/2 as well as eGFP for imaging purposes. Subsequent analyses encompassed evaluations of cell viability and morphological assessments on recipient glioblastoma cells. This revealed growth-limiting effects on the patient-derived primary tumour culture GS.607 with an apparent reduction in total cell count relative to Adenosomes expressing control KIPs.

CONCLUSION: While further studies are needed and ongoing, including Adenosome particle characterizations, mode-of-therapeutic action analysis, safety and efficacy studies for in vivo delivery, our pioneering findings suggest the viability of a new type of (EV-based) gene therapy approach to combat GBM via personalized attack of dysregulated kinase pathways.

Poster Presentations

NLSEV2023-P18: Charlotte V. Hegeman

Modulating binding affinity of aptamer-based loading constructs for efficient EV-mediated CRISPR/Cas9 delivery

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BACKGROUND: The CRISPR/Cas9 toolbox consists of modular nucleases that can be employed to efficiently modify genomic sequences with high specificity. Hence, these systems open up new avenues in the development of gene-editing therapies. However, targeted delivery of the large Cas9-sgRNA ribonucleoprotein (RNP) complexes remains challenging due to immunogenicity, negative charge, and rapid degradation. An approach to overcome these limitations is the use of extracellular vesicles (EVs) as intercellular delivery vehicles. EVs exhibit the natural ability to carry RNA and proteins across biological barriers, and can be engineered to load and deliver biotherapeutic molecules and target specific tissues.

METHODS: To load Cas9-sgRNA RNPs into EVs, sgRNAs containing MS2 aptamers and a fusion protein of CD63 and tandem MS2 coat proteins (MCPs) were expressed alongside Cas9 and VSV-G in HEK293T cells. To study the effect of binding affinity on Cas9-sgRNA delivery, various mutations affecting binding affinity were made in both the interacting sgRNA MS2-hairpin and the RNA-binding domain of the MCP. To separately study the effects of affinity on cargo loading and release, a UV-sensitive photocleavable protein (PhoCl) was included to the MS2-CD63 construct to maximize cargo release. We used a previously published fluorescent stoplight reporter system that can be activated by Cas9- to measure Cas9 RNP delivery (De Jong et al., Nat Commun. 2020).

RESULTS: We confirmed that adaptation of the sgRNAs did not adversely affect their functionality by direct transfection. Comparing Cas9-sgRNA delivery of the modulated sgRNAs revealed that adapting binding affinity highly affects functional delivery (0,5% to 22,2%). A similar effect on functional delivery was seen after modifying the affinity of the RNA-binding domain of the MCP. After UV-treatment, photocleavable MS2-PhoCl-CD63 fusion proteins revealed similar Cas9 delivery for most sgRNAs with varying affinities, indicating that cargo release, and not loading, was a limiting factor in aptamer-mediated Cas9 delivery for most of these sgRNAs.

CONCLUSIONS: Here, we describe a novel way to optimize EV-mediated loading and delivery of Cas9-sgRNA RNPs. Our results demonstrate that EVs are capable of functional Cas9-sgRNA complex delivery and that modulation of binding affinity can be used to increase delivery efficiency.

Poster Presentations

NLSEV2023-P19: Jelle van den Beukel

Stable spike-in counting beads for extracellular vesicle concentration measurements

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INTRODUCTION: There is currently no metrologically traceable way to relate the measured counts of extracellular vesicles (EVs) by flow cytometry to the concentration of EVs. Manual dilution steps of samples cause dilution errors, and flow cytometers have typically a fluctuating, and thus unknown, flow rate. The aim of this project is to test whether potential spike-in counting beads are stable in blood plasma and can be used to track the sample dilution as well as the measured sample volume by flow cytometry.

METHODS: As potential spike-in counting beads we investigated the stability of commercially available polystyrene (PS) and gold beads due to their wide range of available sizes, general applicability, and high refractive index relative to EVs, thereby allowing identification of the beads. The selected beads were: NIST-standard PS beads, PS beads with amine (PS-NH2) and carboxylic acid (PS-COOH) functionalities, and unmodified gold beads. Beads with a diameter of 200 nm were diluted in milliQ water (MQ), Dulbecco's phosphate buffered saline (DPBS), and a 1% plasma solution diluted in DPBS. To study the stability of beads, their concentration was measured by flow cytometry (Apogee A60-Micro) over a time period of 16 hours.

RESULTS: In MQ, PS beads were stable during 16 hours, whereas the concentration of unmodified gold beads decreased 3.1% per hour, probably due to sedimentation. In diluted plasma, none of the tested bead types were stable during 2-16 hours. The concentration of PS beads showed a linear degradation over time and decreased 2.5% (PS), 0.7% (PS-NH2), and 2.5% (PS-COOH) per hour.

CONCLUSION: Commercially available beads of gold and modified and unmodified PS are unstable in plasma diluted in DPBS. This means that spike-in counting beads for EV concentration determination in plasma are currently unavailable and have to be developed.

Poster Presentations

NLSEV2023-P20: Marije Kuipers

Identification of protein properties associated with prostate cancer EVs using machine learning

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INTRODUCTION: Tumor-derived extracellular vesicles (EVs) contribute to metastases and have biomarker potential. To understand and utilize these EVs, in-depth characterization of their content is essential. EV-associated proteins have been identified and quantified by many studies. A recent study showed that EV association can be predicted from protein sequence by machine learning models. Feature importance analysis showed that certain types of post-translational modification sites strongly influence this EV association. However, that model used datasets including EVs from various cell types and body fluids. Here we investigate whether EV proteins from prostate cancer cells can also be predicted using machine learning and whether these differ in a more metastatic derivative.

METHODS: EVs from PC3 and the more invasive derivative PC3M cells were purified using differential centrifugation and density gradient centrifugation. Peptides from lysed cells and purified EVs were obtained by in-gel trypsin digestion and measured by mass spectrometry. Proteins were annotated in MaxQuant and processed in Perseus to compile EV and non-EV protein datasets. For predicting EV-associated proteins, random forest algorithm models were used with 95 selected protein features that included sequence-based features and curated annotations.

RESULTS: EV-associated proteins of PC3 and PC3M were predicted with an area under the curve (AUC) of 0.75±0.03 and 0.74±0.02, respectively. Prediction AUCs increased to 0.77±0.02 (PC3) and 0.79±0.02 (PC3M) when using the top 40% most abundant EV proteins, indicating that more abundant proteins in EVs are easier to predict by the model. Feature analysis showed that palmitoylation, transmembrane regions, and glycosylation were the most important characteristics of EV-associated proteins for both PC3 and PC3M. However, we also observed differences between PC3 and PC3M in protein features important for EV association. We hypothesize that features more prevalent in PC3M EV proteins could link with invasiveness.

CONCLUSION: Machine learning trained on comprehensive EV protein datasets can help elucidate new patterns in EV-associated proteins unique for specific cell types and identify specific protein properties to aid in the search for new biomarkers and treatments. Here we used this strategy to compare EV proteins between PC3 and PC3M cells, potentially revealing mechanisms that increase metastasis via EVs.

Poster Presentations

NLSEV2023-P21: Lei Zhu

Examples of assay controls to confirm that flow cytometry events are extracellular vesicles

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INTRODUCTION: The most commonly used method to characterize single immunolabeled EVs is flow cytometry (FCM). Events measured by FCM are not necessarily associated with EVs, but could be induced by aggregated antibodies or fluorophores, background noise, labelled non-EV particles, or micelles. To confirm that events are associated with EVs, the MIFlowCyt-EV framework and a new compendium about EV FCM recommend to perform assay controls. However, scientific literature lacks experimental data of EV FCM assay controls. Therefore, the goal of this work is to provide the first detailed explanation on the purpose, expected outcomes, and problems and solutions that are specific for each assay control.

METHODS: We applied buffer-only controls, buffer with reagents controls, unstained controls, isotype controls, single-stained controls, fluorescence-minus-one controls, procedural controls, serial dilution controls, detergent treatment controls, pre-blocking controls, positive controls, and negative controls using an Apogee A60-Micro flow cytometer operating at a flow rate of 3.0 µL/minute and using side scatter triggering at a wavelength of 405 nm. As test samples, we used DPBS, human plasma samples, human urine samples, an outdated platelet concentrate, and cell culture supernatant containing EVs. As test reagents, we used CD61-PE, CD235a-APC, CD235a-FITC, and CD45-APC.

RESULTS: For each assay control, we present both calibrated and uncalibrated data to illustrate the expected outcomes, as well as the outcomes that should be avoided.

CONCLUSIONS: Assay controls in EV FCM confirm single vesicle EV detection and improve the reliability of EV FCM data. Therefore, assay controls are an essential aspect of EV FCM experiments and their results should always be reported.

Poster Presentations

NLSEV2023-P22: Jari Verbunt

In vitro methods for the characterization of inflammatory potential of bacterial membrane vesicles

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INTRODUCTION: The intestinal microbiota play a pivotal role in human health. In people with obesity and type 2 diabetes (T2D) the composition and functionality of the gut microbiota is often altered, with profound implications for host substrate and energy metabolism. Importantly, gut bacteria produce bacterial membrane vesicles (bMVs) containing bacterial metabolites, nucleic acids and toxins. These bMVs can induce low grade inflammation, a hallmark of obesity and T2D. In this work we studied the inflammatory potential of bMVs in stimulating human adipocytes and THP-1 derived macrophages.

METHODS: bMVs derived from E. coli bacterial monoculture or fecal matter were isolated using (ultra)filtration and size exclusion chromatography (SEC), followed by quantification through nanoparticle tracking analysis (NTA). THP-1 monocytes were differentiated into macrophages and stimulated with various bMV concentrations or lipopolysaccharides (LPS) for 24 hours. Human adipose tissue-derived stem cells (ASCs) were differentiated into adipocytes and treated with LPS for 24 hours, with or without 24 hours of pretreatment with feces-derived bMVs. Expression levels of IL-1β, IL6, and TNFα were quantified using quantitative polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA).

RESULTS: In THP-1 macrophages, proinflammatory gene expression increased proportionally with E. coli bMV concentrations ranging from 10^5/mL to 10^8/mL. For LPS, the clearest dose response effects were observed in IL6 expression. Adipocytes pre-stimulated with feces-derived bMVs, followed by wash-out and LPS stimulation, exhibited enhanced proinflammatory gene expression compared to LPS stimulation alone, indicating a preconditioning effect by these bMVs. Preliminary data suggests that in vitro stimulation (in THP-1 cells and pretreatment (in adipocytes) using fecal bMVs from individuals with BMI ≥25 induced a more robust proinflammatory response than bMVs from individuals with BMI <25.

CONCLUSIONS: Bacterial membrane vesicles add complexity to host-microbe interactions, and their inflammatory potential underlines their relevance in the onset and progression of metabolic diseases. This study successfully employed differentiated human cells to characterize bMVs in vitro. Elevated cytokine signaling following (pre)stimulation with high-BMI bMVs implies their potential contribution to tissue inflammation observed in metabolic diseases and obesity.

Poster Presentations

NLSEV2023-P23: Bram A. Tuinte

The role of cell specificity in the extracellular vesicle mediated spreading of picornaviruses

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INTRODUCTION: Extracellular vesicles (EVs) from naked virus-infected cells can contribute to virus spreading by transmitting infectious virus particles. Longstanding questions in the field are whether EV-enclosed viruses are more or less infectious than their naked counterparts, and whether the composition and functional properties of EV-associated viruses are cell type- and virus type-dependent. Picornaviruses are naked single-stranded RNA viruses that can infect multiple tissues and might therefore be enclosed in different EVs depending on the infected cell type. Here, we developed a luciferase virus-based method for quantitative comparison of the virus-spreading capacity of EVs from different infected cell types.

METHODS: We used two luciferase picornaviruses, encephalomyocarditis virus (EMCV) and coxsackievirus B3 (CVB3), which encode luciferase in their viral genome. Translation of each viral RNA results in production of a luciferase protein. We additionally used 3 different cell types, Hela-R19 (our standard cell model), Huh7 (hepatocyte-like) and U87 (glial-like). EVs of infected cells were concentrated using differential (ultra)centrifugation and separated from naked virus via isopycnic density centrifugation. Western blot analysis was performed to assess induction of EV release upon infection. Infections were performed using preparations of virus-induced EVs or purified luciferase virus stocks, which were normalized to equal input of viral RNA, determined by RT-qPCR. We quantified infection efficiency of naked- and EV-virus by measuring luciferase production during the course of infection by chemiluminescent detection.

RESULTS: Luciferase-encoding EMCV and CVB3 can infect Hela-R19, Huh7 and U87 cells and can be used to quantify the kinetics and levels of viral replication in these cells. In all three cell types, we observed that EMCV and CVB3 increased production of EVs and EV-enclosed viruses. Purified EVs of all three cell types infected with luciferase viruses could propagate infection and induce measurable luciferase signals in different recipient cells.

CONCLUSION: Luciferase viruses can be used as a tool to quantify efficiency of EV-mediated picornavirus spreading in different EV-donor and recipient pairs. Our search for variations in EV-mediated infection efficiency between cell types could provide information on EV-associated molecules that influence EV fusion with target cells, pro-or antiviral effects of EVs or virion numbers enclosed per EV.

Poster Presentations

NLSEV2023-P24: Busra Kuloglu

A Vascular Voyage: VEGFA isoforms piggyback on distinct Extracellular Vesicles populations in health and pathology

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INTRODUCTION: Vascular endothelial growth factor A (VEGFA) is a key player in tumor-associated angiogenesis and is frequently upregulated in cancer. Recent studies have shown the presence of VEGFA on the surface of small CD63-positive extracellular vesicles (sEVs) derived from cancer cells. EV-bound VEGFA retains its angiogenesis-stimulating capacity and has a dramatically improved half-life, while recognition and therefore neutralization capacity of Bevacizumab, an anti-angiogenesis drug that is part of first-line anti-cancer treatment, is severely reduced. Interestingly, the VEGFA-165 and VEGFA-189 isoforms are differentially expressed in cancer compared to non-cancer conditions. Recent findings in our lab revealed that, in contrast to the more physiological VEGFA-165 isoform, cancer-associated VEGFA-189 markedly boosts its own secretion and sorting to exosomes in a seemingly auto-amplificatory loop. We therefore set out a comprehensive analysis of the intracellular trafficking of the different VEGFA-isoforms and their presence in the broader landscape of EVs.

METHODS: Here, we employ pH-sensitive optical reporters to visualize real-time VEGFA-positive exosome release at the plasma membrane (PM) via TIRF Microscopy. Expansion Microscopy and Transmission Electron Microscopy (TEM) were used to analyze both intracellular trafficking and secreted vesicle populations.

RESULTS: Exploration of VEGFA isoform trafficking through the conventional Golgi secretory route marked by CD9 and VAMP2 and the Late-Endosomal route marked by CD63, revealed that VEGFA-165 showed pronounced preference for the conventional secretory pathway whereas VEGFA-189 is associated more strongly with the late-endosomal pathway. Notably, co-secretion of VEGFA-165 with CD9 exhibited distinct differences in the signal dynamics at the PM compared to VEGFA-189 co-secreted with CD63. Utilizing Expansion Microscopy, we observed VEGFA-189 predominantly resides within CD63-positive subcellular compartments, whereas VEGFA-165 accumulates in proximity to the PM. Lastly, we conducted isoform-specific assessment of VEGFA presence on isolated EVs by Transmission Electron Microscopy (TEM), where we demonstrated that VEGFA-165 carrying EVs show great variability in the size range (50-800 nm), while VEGFA-189 carrying EVs were predominantly found at the sEV range.

CONCLUSION: Our study indicates distinct sorting mechanisms for VEGFA-isoforms onto different sub-populations of EVs marked by different tetraspanins. These findings may have significant implications for understanding angiogenesis and cancer biology, offering potential targets for therapeutic interventions.

Poster Presentations

NLSEV2023-P25: M.H.S. Dekker

Urimon, periodic miRNA profiling in urine for early disease detection

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INTRODUCTION: Liquid biopsies, as a source of biomarkers, are promising for developing widely applicable early disease detection methods, which are needed to improve chances of cure and reduce health care expenses. Based upon early work and literature, we use periodic microRNA profiling in urine to develop a personalized multi-disease early detection test. For this we conduct the Urimon study: 10.000 initially healthy people >45 years of age periodically donate blood (yearly) and urine (trimonthly) over the course of 2-6 years. ~8% develop a serious disease (cancer, cardiovascular, neurodegenerative) during participation and their sample series thus capture the early stages of disease.

METHODS: Mid-stream, morning urine is home-collected in a preservative-containing cup. Plasma is collected in EDTA/PAXgene or Norgen preservative tubes. Processing to plasma and cell free urine is done in a central lab and samples are stored at -20 °C (urine) or –80 °C (plasma) in the Stibion biobank (available to all researchers). As the Urimon study progresses, disease cases accumulate as in the Dutch society. Those sample series are thawed, and microRNAs are isolated. microRNA profiles are generated by IsoSeek (Quantitative NGS) and analyzed statistically (LASSO) to discover and test microRNA panels that can detect and specify disease within these longitudinal sample series.

RESULTS: The first cohort analyzed included 89 participants, of which 56 donated more than 4 samples before disease was diagnosed. In total 429 samples were analyzed from healthy donors (10), 6 types of cancer patients (28) and 4 types of cardiovascular disease (30). Statistical analyses showed that microRNA profiles were donor-specific and certain panels indeed distinguished donors that stay healthy from those that develop disease, already before diagnosis. Specific diseases could be distinguished as well, but at varying specificity. Determining the contribution of personalized/longitudinal profiling to early detection remains challenging.

CONCLUSION: Early detection by urinary microRNA profiling for a number of cancers and cardiovascular diseases was possible. For validation the size of the sample cohort needs to be increased and apparent noise in microRNA profiling requires more attention. The Urimon sample series provide a valuable resource for early disease biomarker R&D (microRNA or other).

Poster Presentations

NLSEV2023-P26: Tingting Chen

Transferring mitochondria rescues neuronal cells from ferroptosis

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INTRODUCTION: Ferroptosis is a form of oxidative cell death that involves mitochondrial damage and that has been frequently linked to neuronal cell death in neurodegenerative diseases. Previous studies demonstrated that preventing mitochondrial dysfunction can rescue cells from ferroptotic cell death. However, the complexity of mitochondrial dysfunction and the timing of therapeutic interventions make it difficult to develop an effective treatment strategy against ferroptosis in neurodegeneration conditions.

METHODS: In the present study, we employed mitochondrial transplantation to neuronal cells as a novel therapeutic approach to prevent ferroptotic cell death induced by glutamate, erastin, or RSL3. We checked cell metabolic activity by MTT assays. The incorporation of mitochondria was observed by live imaging. Cell death and cellular oxidative stress level were assessed via specific dyes followed by flow cytometry. The oxygen consumption rate was determined through seahorse analysis. In addition, we employed microfluidic devices which enable us to separate the neurites of primary neurons from their cell bodies. The neurite area after different treatment was measured by immunofluorescence and quantified by ImageJ-Plug-In NeuronJ.

RESULTS: Our data indicate that isolated exogenous mitochondria are incorporated into both healthy and ferroptotic immortalized hippocampal HT-22 cells and primary neurons. The mitochondrial incorporation into ferroptotic cells was accompanied by increased metabolic activity and cell survival through attenuating lipid peroxidation and mitochondrial superoxide production. Further, the function of mitochondrial complexes I, III and V of mitochondria in ferroptotic host cells have been supported by the transplanted mitochondria. Similarly, we have also captured the internalization of exogenous mitochondria in host mouse primary neurons; these internalized mitochondria are found to effectively preserve the neuronal networks when challenged with RSL3. The administration of exogenous mitochondria into the axonal compartment of a two-compartment microfluidic device induced mitochondrial transportation to the cell body, which repaired the fragmented neuronal network in ferroptotic primary neurons.

CONCLUSION: Our findings propose mitochondria transplantation as a promising therapeutic approach for protecting neuronal cells from ferroptotic cell death.

Poster Presentations

NLSEV2023-P27: Elly Soltani

Distinct sorting of cancer specific VEGFA-isoforms on EVs might underly chronically activated angiogenesis in colorectal cancer

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INTRODUCTION: Vascular Endothelial Growth Factor A (VEGFA) is an angiogenic factor secreted in response to hypoxic conditions to stimulate blood vessel formation, restoring normal tissue homeostasis. In cancer, however, VEGFA secretion is linked to abnormal and disorganized blood vessel formation. Interestingly, VEGFA isoform VEGFA-189 is more prevalent in cancer, while VEGFA-165 is more common in non-cancerous states. Although VEGFA was traditionally thought to follow the conventional secretory pathway, recent discoveries have unraveled its presence on the surface of Extracellular Vesicles (EVs), dramatically extending its lifespan. Yet, the process of loading VEGFA onto EVs and its relevance to aberrant vessel formation observed in cancer remains unclear.

METHODS: We studied the intracellular trafficking of both VEGFA-isoforms using TIRF- and spinning-disk microscopy. EVs and their tetraspanin composition were assessed by Electron Microscopy (EM) and ten-fold robust expansion microscopy (TREx), which allows a volumetric view of VEGFA an tetraspanin distribution on EV surfaces.

RESULTS: We found that the VEGFA-165 isoform preferentially takes the conventional secretory pathway and is mainly present on larger EVs that likely result from PM budding. Conversely, VEGFA-189 is detected mostly on smaller EVs and tightly linked to the (multivesicular) endosomal pathway.
Strikingly, we found VEGFA-189 to stimulate its own secretion by reorganizing the endosomal system via nuclear translocation of the transcription factor EB (TFEB). Mechanistically, VEGFA co-receptor NRP1, upregulated in colorectal cancer, seems to play a major role in both the induction of VEGFA-189 export and its routing in the late endosomal pathway.

CONCLUSION: We found that, in contrast to VEGFA-165, cancer-associated isoform VEGFA-189 reorganizes the endosomal system and boosts its own secretion on exosomes. Combined with the upregulated levels of NRP1 and VEGF-Receptor 2 (VEGFR2) observed in various cancers, this likely creates an auto-amplificatory loop potentially explaining the aberrant vascular growth observed in cancer.

DISCUSSION: VEGFA is seen as a promising target for cancer treatment, increasing our understanding of how VEGFA is loaded onto EVs and its role in tumor progression will hopefully provide for interesting therapeutic targets. Likewise, the insights and techniques applied in this study might broaden our knowledge of EV protein-corona cargo loading in general.