PhD thesis

The role of membrane nanotube networks in the communication of immune cells

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UNIVERSITY OF PÉCS
MEDICAL SCHOOL

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INTRODUCTION

The transfer of information between cells contributes to the perception of stimuli from the environment, facilitates rapid communication and coordinated functioning of cells, and is indispensable for the development, survival, tissue regeneration, and maintenance of the physiological state in higher organisms. To ensure this kind of information exchange, many mechanisms and structures have been developed. These include, for example, cell junctions [1–4], which directly connect cells in contact with each other and extracellular vesicles [5,6] as well as filopodia [7], which ensure communication between cells located far away from each other. A membrane nanotube (NT) is a dynamic, tube-like, cylindrical cell extension described in 2004 [8] that physically connects cells that are not in contact with each other. The presence of NTs has now been proven *in vitro* [9–11], *ex vivo* [9,12,13], and *in vivo* [14–16]. In addition to eukaryotes, they also occur in archaea [17] and bacteria [18,19].

NTs are built from cytoskeletal filaments. Their main cytoskeletal element is filamentous actin (F-actin), which is essential for their formation and some of their biological functions [8,9]. However, in many NTs, microtubules [9,11] and intermediate filaments [20,21] can also be found. The role of these filaments is still not well understood, but they likely contribute to the stability of NTs and increase their lifespan [20,22].

NTs are similar in many ways to other cell extensions (e.g., lamellipodia, streamers, cytonemes, filopodia [23–25]) and are heterogeneous structures both in terms of their formation and morphology, which makes their identification difficult. Distinguishing them from other cell extensions is not easy because there is currently no specific marker available that would make only NTs visible during microscopic visualisation. Examinations performed with high-resolution microscopes led to the creation of a system of criteria that enables the separation of NTs from other cell protrusions. These morphological characteristics are as follows:

1.) based on experiments on cell cultures, the NTs do not stick to the substrate but instead 'float' in a stretched state between distant cells [8],
2.) they are also able to physically connect two or more cells, thus creating continuity between the cytoplasms of the cells [8,9],
3.) in addition to all this, they carry out intensive transport processes [8].

The exact mechanism of their formation is not known in detail even today. Their formation is basically explained by two models. One theory is based on the so-called actin-driven protrusion, where a filopodium-like extension can elongate through actin elongation and attach to the membrane of another cell (or to a cell extension grown by another cell) [26]. The other is the theory based on cell separation, during which two cells that were previously in

contact with each other separate and then, during their movement in opposite directions, form the membrane bridge that connects them [11]. The biogenesis of NTs can be influenced or aided by many factors, including various stress effects such as temperature changes, hypoxia, serum deprivation, increased glucose concentration, bacterial and viral infections, or UV radiation [27–31]. In addition to these, certain proteins or protein complexes also affect the formation of NTs (e.g., Eps8 [32], I-BAR [33], LST-1 [34], myosin X [35], M-sec [36], CDC42/VASP/IRSp53 [32]); however, the results of experiments aiming to reveal their molecular and regulatory processes remain quite contradictory [37,38].

It was mentioned above that NTs are not only heterogeneous in terms of their formation, but their appearance can also be very diverse. Consequently, we distinguish between open-ended and closed-ended NTs, identifying several subgroups within each. The open-ended ones, which physically connect the cytoplasm of the cells forming them, can be thin (less than 700 nm in diameter, usually containing only actin) [11], thick (more than 700 nm in diameter, containing both actin and microtubules) [11], and so-called individual NTs (formed by the connection and twisting of different NT bundles) [39]. Closed-ended NTs do not actually connect the cytoplasm of the cells [39–41], but sometimes they can be connected to the other cell's membrane by synapses [42].

NTs play a role in the transport of many substances [43], influencing different physiological and pathological processes [29,44]. Their participation has been demonstrated in signal transmissions, such as in calcium signal transduction [45], apoptotic processes [46], and the intercellular propagation of bacteria [11,31] and viruses [28,47] of outstanding biomedical importance. This is interesting because pathogens can hide from the cells of the immune system by moving inside NTs and can also infect cells that are normally not susceptible to the given pathogen [48], thus influencing the outcome of some diseases and the severity of related complications. In addition, NTs may be involved in the development and progression of certain neurodegenerative diseases by transmitting proteins with defective spatial structures, such as prions (e.g., tau [49], alpha-synuclein [49], and huntingtin [50]), thereby influencing the course of Alzheimer's, Parkinson's, and Huntington's diseases [51]. In addition, NTs probably affect the progression of cancer diseases [52] and their resistance to chemotherapy agents [53], in which the exchange of mitochondria between distant cells with the help of NTs may play a role [54]. The transfer of mitochondria between cells is a relatively common phenomenon and can occur through several routes, such as via extracellular vesicles [54] or gap junctions [54]. However, it has also been observed that mitochondria can be transported via NTs in many cases [46], making the study of mitochondrial transport a focal area in NT research [11,55]. This is

how it became clear that the transport of mitochondria via NTs can play a key role, for example, in rescuing damaged cells and improving their energy balance [29]. However, NTs not only take part in the delivery of mitochondria but are also actively involved in the intercellular transport of other cell components (e.g., vesicles, lysosomes, autophagosomes [8,9,56], endosomes [10], endoplasmic reticulum (ER), Golgi [10,28], nucleus [57]). In addition to the above, NTs can also play a role in the regulation and efficient functioning of the immune system [58], and they represent a form of trogocytosis that creates an opportunity for the intercellular transmission of the protein content of immune cells located at great distances from each other [59]. The function of NTs in immune processes (e.g. enhancing the phagocytic capacity of macrophages [58] or activating T-cells [60]) necessitates the reinterpretation of many currently valid and accepted basic immunological concepts [61].

AIMS

Although a significant part of nanotube research has been carried out on the cells of the immune system, very little information is available on the NTs of B-cells, which play a key role in the humoral immune response, especially regarding their functional significance. Despite that B-cells also perform important cellular functions, they play a role in treating pathogens through antigen presentation to T-cells and in activating T-cells, where costimulatory molecules expressed on the surface of B-cells are essential.

It is known that the ability of B-cells to form NTs is influenced by environmental parameters, the maturation state of the cells, the interaction of integrins on their surface with extracellular matrix proteins, and the lipid composition of the cell membrane [9,62]. It is known that most B-cell NTs contain microtubules. At the same time, we have little information about their role within NTs of immune cells [11], and no information at all in B-cells [9]. Simultaneously, there is a strong indication that NTs between B-cells facilitate the transfer of various cellular constituents among interconnected distant cells. However, they may be mediated by molecular mechanisms that have not been identified yet.

In light of these considerations, we pursued answers to the following questions:

1. What is the functional role of NTs that spontaneously form between B-cells? Is there intercellular material transport (microvesicles, cell organelles) through these tubes? We also wanted to characterise the transport properties (direction, speed, movement pattern).
2. Which are the primary motor proteins controlling the transport processes in NTs connecting B-lymphomas?
3. How are the main cytoskeletal polymers (actin filaments, microtubules) spatially organised in the NTs of B-lymphoma cells? Our primary objective was to investigate this using superresolution microscopy and to interpret the observed spatial patterns.
4. How are actin bundles and microtubules oriented during the biogenesis of NTs, and what is the functional role of these cytoskeletal elements in the NTs of B-lymphoma cells?
5. What changes are induced by the interaction with bacterial toxins in the membrane of both immature and mature B-cells, as well as in the formation of nanotubes? Are B-cells able to transport these toxins through NTs?
6. Are B-cells and macrophages acting as antigen-presenting cells to T-cells capable of intercellular transport of the CD86 costimulatory membrane protein via NTs, and by what mechanism does this take place?

MATERIALS AND METHODS

Applied cell lines and their maintenance

In our studies, we utilised the immature 38C13 − B-lymphoma, mature A20 − B-lymphoma (TIB208), and Raw 264.7 – Abelson murine leukaemia virus-induced tumour macrophage (TIB-71) mouse cell lines. They were maintained under sterile conditions in RPMI-1640 medium supplemented with 2 mM ultraglutamine, 1 mM Na-pyruvate, 50 μM mercaptoethanol, 9.8 μM HEPES and 10% FBS. The cells were cultured at 37 °C in the presence of 5% CO_2 in a 25 cm^2 flask. The cells were regularly tested for the presence of mycoplasma, and in our studies, we only worked with mycoplasma-free cultures.

Reagents and dyes

To create an extracellular environment, the Petri dishes with borosilicate bottoms used in the microscope were coated with fibronectin (FN) at a concentration of 10 μg/ml. The cells were labelled either in cell suspension and then transferred to a coated Petri dish, or the cells already attached to fibronectin were labelled.

To examine vesicular transport, cells were labelled with Alexa Fluor (AF) 488 and/or 647 cholera toxin-B (later CTX-B; 40 μg/ml). The labelled cells were maintained in a glass-bottomed, FN-coated Petri dish under thermostatic conditions. MitoTracker Orange CMTMRos fluorescently conjugated dye (50 nM) was used to study mitochondrial transport. The labelled cells were incubated in a coated Petri dish. Specific and selective inhibitors for different motor proteins were used to inhibit vesicular and mitochondrial transport (*Table 1*). The co-occurrence of motor proteins with various cell constituents, the endogenous appearance of CD86, and its possible compartmentalisation in caveolae were investigated using immunofluorescence. AF 568 phalloidin dye was used to label actin, and immunofluorescence was applied for labelling microtubules. The study of the actin and microtubule system in B-lymphoma NTs was performed under live-cell conditions, using different silicone rhodamine-based (SiR actin/SiR tubulin) fluorogenic probes and organic dyes (Live 510/560 tubulin). In each case, the dyes were added to the living cells at a concentration of 1 μM, in the presence of 20 μM verapamil. One major disadvantage of the labelling is that the dye can be difficult to visualise after a few hours and may eventually become undetectable, which is attributed to the dynamic function and active pump mechanisms of B-lymphoma cells. Thus, plasmids were primarily used to study cytoskeletal polymers and their orientation.

Plasmids, transformation, gene silencing and electroporation

To study endogenous actin, LifeAct RFP and GFP, for microtubules mTagRFP-T-Tubulin-6 (http://n2t.net/addgene:58026) and EGFP-Tubulin-6 (http://n2t.net/addgene:56450) [63] plasmids were used. A pmax-GFP vector was used as a control.

The pCMV6-AC-GFP plasmid was used to examine the CD86 immune costimulatory protein, while the pCMV6-AC-GFP vector without insert was used as a control. The plasmids were introduced into the chemically competent TOP10 *E. coli* cells during chemical transformation. The transformed cells were propagated in Luria Broth medium, then plated on agar containing the appropriate antibiotic. Following the growth of resistant colonies, DNA was extracted from the culture using the NucleoSpin Plasmid EasyPure kit. The concentration of the purified plasmids was measured with a Nanodrop One C spectrophotometer.

Fluorescently conjugated small interfering RNA (siRNA) molecules were designed to study the role of kinesin (KIF5B) and myosin VI in mitochondrial transport. The following sequences were used for our experiments (QIAGEN): KIF5B siRNA#3 − target sequence: 5'-CAGCAAGAAGTAGACCGGATA-3', KIF5B siRNA#4 − target sequence: 5'-AACACGAGCTCACGGTTATGC-3', MYO VI siRNA#3 − target sequence: 5' -CAAGTTCAAGACACAATTAAA-3' and MYO VI siRNA#4 − target sequence: 5'-CAGCAGGAGATTGACATGAAA-3'. Due to the low transfection efficiency of the A20 cell line, AF 488 fluorophore was conjugated to the 3' end of the sequences during the design. AF 488 AllStars Negative Control siRNA (QIAGEN), AllStars Mm/Rn Cell Death Control (QIAGEN), and PBS were used in the negative control, positive control, and mock control experiments, respectively.

Plasmids and siRNAs were introduced by electroporation (using Amaxa Nucleofector 2b or 4D devices). Microscopic visualisation was achieved 24 hours post-transfection for plasmids and 72 hours post-transfection for siRNAs. In the case of heterocultures, a 1:1 mixture of cells electroporated with different plasmids was used.

Microscopy

For microscopic visualisation, 0.17 mm thick Petri dishes with borosilicate bottoms, coated with fibronectin (FN), were used in all cases. The movement of cell organelles and the biogenesis of NTs were investigated under live-cell conditions (37 °C, 5% CO_2). Mitochondrial transport and growth of NTs were monitored using a Zeiss laser scanning confocal microscope (LSM-710). The images were taken with a 63× magnification oil immersion objective lens (NA.: 1.4), using 1 Airy unit. The morphological changes of the NTs were examined in the

widefield mode of the Zeiss Elyra S1 SIM microscope, with 40× objective magnification (NA.: 0.75). Motor proteins and vesicular transport were visualised with an SR-SIM microscope, using a 63× objective magnification (oil immersion lens, NA.: 1.4) and 5 grid rotations. Optical slicing (Z-stack) was used during microscopic imaging, with steps of 0.67 µm for LSM and 0.80 µm for SIM. When examining the transport processes, the movement of cell organelles was followed for 40 cycles, with a time interval of 8 seconds between each cycle. The formation of NTs and the examination of the orientation of cytoskeletal filaments were monitored for 6-12 hours, at intervals of 5 and 10 minutes, respectively. A STEDYCON microscope at 100× magnification (oil immersion, NA.: 1.45) was applied to reveal the positional relation of actin and microtubules in NTs.

Statistical analysis

Microscopic image analysis was performed with Fiji, Imaris 8.2, Zen Black 2.1 SP3, and Zen Blue 2.3 software. The ratio of mobile vesicles in NTs was calculated using the formula $mobile\ vesicle\ ratio\ (\%) = \frac{number\ of\ mobile\ vesicles\ in\ NT}{total\ number\ of\ vesicles\ in\ NT} * 100.$ A constrained iterative algorithm was used for the deconvolution. The diameter and length of NTs were determined as previously published [64]. The circularity of the cells was determined using the formula $f_{circ} = \frac{4\Pi A}{P^2}$ (where A: area of the cell, P: perimeter of the cell).

Statistical analyses were performed using Origin 2020 and IBM SPSS v26 software. The Kolmogorov-Smirnov test was used to determine the normal distribution of the data. In the case of a normal distribution, an ANOVA test was performed. For data showing a non-normal distribution, post-hoc tests were performed using the Kruskal-Wallis test to compare the treated and control samples. In the case of a normal distribution, Student's t-test was used to confirm the test and determine the type II error. For data analysed with Kruskal-Wallis, the Mann-Whitney U test was used to confirm the significance values suggested by the Kruskal-Wallis analysis. The significance level was determined at $p < 0.05$.

RESULTS

Vesicular transport between B-lymphoma cells

The ability of B-cells to form NT is closely related to the lipid composition of their cell membrane, particularly the GM1/GM3 ganglioside content. The membrane of immature B-cells is extremely poor in gangliosides; therefore, NT is not formed between them, in contrast to mature B-cells that are rich in lipid rafts [62]. CTX-B isolated from the bacterium *Vibrio cholerae* can bind to membranes rich in GM1/GM3 gangliosides and lipid rafts. By treating with CTX-B, we modelled an everyday *in vivo* event, such as the interaction of bacterial toxins with B-cells. Fluorescently conjugated CTX-B was internalised into the cytoplasm of the cells after binding to the gangliosides in the cell membrane. As a result, the mature B-lymphoma (A20) cells formed microvesicles with a diameter of 100-1000 nm, which became visible within 2 minutes after the addition of CTX-B, and their number saturated after 30-40 minutes. (No such effect was observed in immature B-cells (38C13)). The formed vesicles moved both uni- and bidirectionally in the NTs between the B-lymphoma cells and were sometimes even exchanged between the cells interconnected by NT. The speed of the mobile vesicles turned out to be quite heterogeneous (*Table 1*); their movement was not necessarily continuous, but sometimes seemed to alternate between active and passive sections. Based on the experiments using selective and specific inhibitors for motor proteins often identified in transport processes, this phenomenon is primarily mediated by the actin-dependent myosin II motor protein, whose inhibition not only caused a significant decrease in transport speed but also changed the movement pattern of the vesicles. However, inhibition of kinesin, myosin V and VI had the opposite effect and caused a significant increase in movement speed (*Table 1*), suggesting that these proteins may also play a role in the transport of vesicles through NTs. The results of our transport inhibition experiments were also confirmed by colocalisation studies, showing a positional relationship between vesicles and myosin II, as well as sometimes kinesin, myosin V and VI.

Mitochondrial transport between B-lymphoma cells

In addition to vesicular transport, we observed intensive uni- and bidirectional mitochondrial transport between B-lymphoma cells. This transport appeared less regular, characterised by alternating active and inactive phases clearly delineated. During the active phase, mitochondria exhibited saltatory movement, while in the inactive phase, they remained stationary. The average speed of mitochondrial transport significantly differed from that observed for vesicles (*Table 1*). We hypothesised that a more complex process is behind

mitochondrial transport. Similar to the investigation of vesicular transport, this process was examined using specific inhibitors for each motor protein. According to our findings, the kinesin and myosin VI proteins significantly decreased mitochondrial velocity and path length within NTs, also altering their motion trajectory. At the same time, the inhibition of dynein, myosin II and V resulted in a significant increase in the rate of mitochondrial transport (*Table 1*), and the movement trajectories of the organelles indicated a more uniform transport. The role of kinesin and myosin VI in mitochondrial transport was also proven by gene silencing, the decrease in the expression of both motor proteins slowed down the movement of mitochondria (*Table 2*).

Treatment	**Target protein**	**Ratio of mobile vesicles (%)**	**Velocity of vesicular movements (nm/sec) and sample size (pcs)**	**Velocity of mitochondria movements (nm/sec) and sample size (pcs)**
-	-	83 ± 24.27	15.00 ± 8.57 69	28.16 ± 11.42 40
inhibition of microtubule-based motor proteins				
Nocodazol 10 és 20 µM	microtubules	82 ± 26.06	25.52 ± 18.42 *↑ 53	21.06 ± 7.65 *↓ 40
Ciliobrevin-D 20 µM	dynein	73 ± 36.03	15.73 ± 9.58 64	33.51± 18.42 ↑ 38
Ispinesib 15 µM	kinesin	76 ± 31.42	30.15 ± 18.26 *↑ 60	20.86 ± 12.89 *↓ 40
inhibition of actin-based motor proteins				
paraNitro- blebbistatin 40 és 20 µM	myosin II	13 ± 20.12 *↓	5.52 ± 4.24 *↓ 67	31.89 ± 12.43 ↑ 40
MyoVin-1 30 µM	myosin V	84 ± 19.71	42.40 ± 24.03 *↑ 60	33.63 ± 22.28 ↑ 38
TIP 30 µM	myosin VI	66 ± 23.16	31.73 ± 15.28 *↑ 63	23.19 ± 14.17 *↓ 38
Ispinesib-TIP 15 µM-30 µM	kinesin- myosin VI	-	-	12.93 ± 4.82 *↓ 46

Table 1. Summary of the results of our transport inhibition experiments

*The significance level (*) was determined at $p < 0.05$, *↓: the significant decrease, *↑: the significant increase, ↑: the small increase in each treatment compared to the control. The table shows the mean ± SD values. The TIP is the abbreviation of 2,4,6-Triiodophenol.*

siRNA	Target protein	Velocity of mitochondria movement (nm/sec)	Sample size (pcs)
-	-	36.27 ± 20.59	40
KIF5B siRNA#3	kinesin 5B	23.58 ± 12.62 *↓	40
KIF5B siRNA#4	kinesin 5B	17.11 ± 6.62 *↓	40
MYO VI siRNA#3	myosin VI	23.57 ± 13.47 *↓	40
MYO VI siRNA#4	myosin VI	18.21 ± 8.15 *↓	40

Table 2. Summary of the effect of kinesin and myosin VI silencing on mitochondrial transport rate
*The significance level (*) was set at $p < 0.05$. *↓ indicates a significant decrease compared to the control using different target sequences. The table shows the mean ± SD values.*

Transport of immune costimulatory protein CD86 through the NT of different APCs

In addition to studying the transport of cell constituents, we were also interested in whether NTs created between immune cells are capable of transporting costimulatory proteins and transferring them between cells, as they play an important role in the development of the immune response. During costimulation, different costimulatory proteins (CD86/B7-2 and CD80/B7-1) expressed on the surface of APCs bind to the T-cell receptor and transmit the antigen to the T-cells. The expression level of costimulatory proteins differs between different APCs [65,66], it is lower in macrophages than in B-cells [66], and their current level in the membrane of APCs determines the ability of these cells to activate helper or cytotoxic T-cells and thus the efficiency of the subsequent immune response. We found that the appearance of endogenous and exogenous CD86 in B-lymphoma cells is different: the endogenous protein showed a homogeneous distribution in the cytoplasm, and the exogenous protein was mainly localised in the cell membrane. In contrast, in macrophages, endogenous and exogenous CD86 colocalised with each other, and their appearance was related to the cell membrane. This observation is not surprising, since after their synthesis in the endoplasmic reticulum, CD86 molecules are packaged in vesicles and transported from the cytoplasm to the cell membrane, moreover, the expression of CD86 is significantly lower in macrophages. Although, based on our video recordings, the transport of CD86 was observed in the NTs of both cell types, it showed a different pattern. While CD86 molecules in B-lymphoma cells predominantly moved within the membrane of NTs, in certain macrophages, they were transported as large, elongated structures within the lumen of NTs. Additionally, exogenous CD86 in macrophages sometimes exhibited an appearance resembling microvesicles in the cytoplasm. Based on our results, CD86 molecules via NTs are not transported in caveolae or GM1/GM3-containing vesicles.

Examination of the cytoskeletal elements of NTs between B-lymphoma cells

When inhibiting microtubule polymerisation, NTs changed morphologically. When nocodazole was applied at a high concentration (20 μM), these changes occurred suddenly, 27% of the tubes were already ruptured at the initial stage of the treatment, making microscopic visualisation difficult. When the concentration of the inhibitor was halved (10 μM), a much higher proportion of NTs remained intact (12.5% of them were torn). As a result of microtubule depolymerisation and fragmentation caused by nocodazole, the diameter of NTs significantly decreased by the 5th minute of treatment. Approximately 20 minutes later, the thickness of the tubes did not change significantly. The treatment did not affect the length of NTs or the ellipticity of the cells.

Based on our results, thin NTs containing only actin have a shorter life span, they disintegrate and break after 20-30 minutes. On the other hand, the thicker ones, which also contain microtubules, can last for hours, but at the same time they develop more slowly, and in these NTs, microtubules appear already at the beginning of NT formation. It was determined that the NTs of B-lymphoma cells are also diverse based on the pattern of the cytoskeletal filament systems found in them. One group of NTs contains only the actin network from one of the cells involved in tube formation, while in another group, the actin networks from both cells can be observed. These networks are sometimes separate but can also partially overlap. Similar to actin, different growth patterns and orientations were observed for microtubules. In some of the NTs, the microtubules originate from only one of the tube-forming cells, but sometimes they are not found along the entire length of the tubes. Microtubules of the receding cell grow into these protrusions. In some cases, microtubules can originate from both cells involved in NT formation and can be found along the entire length of NTs, overlapping each other.

A brief summary of our results

In B-lymphoma NTs the actin filaments are positioned beneath the membrane that covers the NTs, while the microtubule bundles predominantly run longitudinally along the centre of the NTs, probably because due to their high persistence length, microtubules exhibit sensitivity to cell geometry and align axially, leading to the arrangement of microtubule networks along straight structural elements [67]. Moreover, microtubules sometimes exhibit a twisted pattern. The cytoskeletal composition of NTs can be formed with the participation of both cells involved in the formation of the NT, cytoskeletal filaments from different cells can even partially overlap in the NT. Transport processes occurring within B-lymphoma-cell NTs can be facilitated by both actin- and microtubule-mediated mechanisms. Myosin II transports microvesicles towards

the plus end of actin, whose bidirectional transport is most likely due to overlapping actin networks. We hypothesise that microvesicles may interact simultaneously with multiple motor proteins (e.g., dynein, kinesin, myosin V, and VI) during their inactive phase of movement. The bidirectional movement of mitochondria is accomplished through the cooperation of two motor proteins: kinesin transports the mitochondria towards the plus end of microtubules, whereas myosin VI conveys them in the opposite direction along actin filaments. Mitochondrial movement is highly irregular and characterised by frequent saltatory (active) and stationary (inactive) phases. We suppose that dynein, myosin II, and myosin V motor proteins are responsible for the anchoring or docking of the mitochondria along the cytoskeleton of the NT, contributing to the stationary phase of the transport. The process of mitochondria transport enters a resting phase upon the binding of motor proteins responsible for transportation and the corresponding docking motor proteins, thus maintaining their association with the filament. The continuous movement of mitochondria becomes apparent when the docking motor proteins are released. In B-lymphoma cells, CD86 transport occurs at the surface of the membrane.

DISCUSSION

Our research group has been studying NTs between immune cells for approximately 7 years. In my thesis, I investigated whether the NTs of mature B-lymphoma cells contribute to enhancing the immune response and if they function as effective cellular extensions. Additionally, we aimed to characterise vesicular and mitochondrial transport, which appears to be more prevalent based on previous results, and to explore the underlying molecular processes.

We have shown that the transport of vesicles and mitochondria within the NTs is controlled by cytoskeletal proteins. Based on our results, we assume that multiple phenomena may underlie the regulation of transport processes. For example, the stress response that occurs after the addition of an inhibitor, where cells assist each other with more intensive transport processes. However, the observed results may also suggest a complex regulatory system in which cell organelles are attached (docked) to cytoskeletal filaments by specific motor proteins, leading to irregular movement. If the activity of the docking motor proteins is blocked, their function is impaired, preventing the cell organelles from attaching to the cytoskeleton. Thus, cell constituents are released from the bound state, leading to an increase in transport speed and a change in the movement pattern, resulting in more uniform movement of the transported organelles [68]. Similar docking phenomena described in neurons can contribute, for example, to the proper distribution of mitochondria along long axons, ensuring local energy supply to axonal areas with high energy consumption [46,68–70]. Based on the experimental results, we believe that in microvesicular transport, kinesin, dynein, myosin V and VI, and in mitochondrial transport, myosin II, V, and dynein can act as docking proteins responsible for the irregularity of transport and the alternation between active and inactive phases. The understanding of the background of transport processes is further complicated by the fact that both actin- and microtubule-based motor proteins can bind to the same cargo. This results in the cargo switching between motor proteins of the two cytoskeletal systems during its transportation [68], contributing to the irregular movement of cell constituents. Furthermore, due to electrostatic interactions, microtubule-based motors may be connected with actin and myosins with microtubules, at least *in vitro* in the case of kinesin and myosin V [71]. This highlights the complexity of organelle transport processes, contributing to the successful transport of the cargo.

The transport processes through the NTs enable coordinated cell functioning and rapid communication between cells. Cells connected by NTs can monitor each other's energy status and compensate for energy deficits by transferring mitochondria through NTs. Mitochondria are energy-producing cell organelles essential for all immune cells. During their operation,

various reactive oxygen species (ROS) are generated, which help keep immune cells alert. ROS influence immune cell differentiation, proliferation, regeneration, and the fate and survival of B-cells. Additionally, ROS enhance the activation of the adaptive immune response and the ability of B-cells to produce antibodies [72–74]. The mitochondrial transport between B-cells mediated by NTs can therefore prevent cell death, influencing the homeostasis of B-cells. Mitochondria are therefore not only essential for the adequate energy supply of immune cells, but can also induce immune responses [73].

Cytoskeletal elements are essential for the formation and function of NTs, and they also contribute to the creation of mechanically more stable structures [8,9,11,20]. A cytoskeletal polymer essential for the formation of NTs is actin [9], whose heterogeneous arrangement enables bidirectional microvesicular transport and likely contributes to the flexibility of NTs [75]. Although microtubules are not necessary for the formation of NTs [9], based on our current results, they are essential for the functioning of NTs in B-lymphoma cells (e.g., through the transport of mitochondria) and for increasing the stability of NTs, ensuring their longer lifespan. It is known that NTs containing microtubules can carry out more intensive transport processes. This is supported by the fact that microtubules exhibit a stiffness that is three orders of magnitude greater than that of actin [76] and possess a significant persistence length, which allows them to retain their shape and anisotropy [67]. As a result, NTs containing microtubules have a longer lifetime compared to those containing only actin [77]. Furthermore, the force generated along axons (which are similar in appearance to NTs) provokes the stabilisation of the microtubules within these protrusions, leading to their decreased turnover. This, in turn, facilitates their accumulation and promotes the transport processes mediated by them [29,78]. This is particularly notable as T lymphocytes, which lack microtubules entirely, do not exhibit similar transport characteristics [40]. Additionally, the growing end of microtubules under tension tends to elongate, potentially decreasing microtubule depolymerisation rates, thus contributing to the mechanical stability of NTs [79,80]. Our findings support that the cytoskeleton provides structural support and regulates NT dynamics, ensuring the proper functioning and durability of these cellular protrusions. Nevertheless, further investigation is needed to precisely understand the mechanical and elastic properties of NTs.

B-cells, which can present antigens themselves, produce antibodies and work closely with cells of the innate immune system, as well as other professional APCs and T-cells. During costimulation, APCs are able to present antigens to T-cells with the participation of various costimulatory proteins, while becoming activated themselves. Based on our results, macrophages and B-lymphoma cells can effectively deliver costimulatory proteins (e.g., CD86)

through NTs. This suggests the existence of a new immunomodulatory pathway that enables the direct transfer of immunoregulatory molecules between cells, thereby contributing to the development of a more effective T-cell activation and immune response against various foreign substances to the body.

CONCLUSION AND PRESENTATION OF NEW RESULTS

Our results may enhance the understanding of the functional significance of NTs, supporting future efforts to develop therapeutic applications of NTs. Our studies are pioneering in both national and international NT research. Our main results, considering the objectives, are as follows:

1. We found that NTs between B-lymphoma cells are highly active communication channels and can play an important role in regulating certain immune processes through the transport of microvesicles, mitochondria, and the CD86 costimulatory membrane protein.
2. We proved that the actin filaments in the nanotubes of B-lymphoma cells run directly beneath the cell membrane and provide the framework for NTs.
3. We found that the microtubules are located in the lumen of NTs, primarily in their central part. They are mostly parallel, although they can sometimes be twisted. They can increase the stability and lifetime of NTs.
4. We revealed that the cytoskeletal composition of NTs can be formed with the participation of both cells that play a role in NT formation, and filaments from different cells may even partially overlap within the NT. Consequently, the arrangement of cytoskeletal filament systems contributes to the bidirectional transport processes observed in NTs. The transport processes taking place in the nanotubes of B-lymphoma cells can be facilitated by both actin and microtubule-mediated mechanisms.
5. B-lymphoma cells exhibit intense vesicle formation in response to bacterial toxin binding on the cell surface. These vesicles originate from the plasma membrane and can be transported bidirectionally through NTs, allowing for their exchange between cells connected by NTs. Microvesicles are transported through NTs assisted by the actin-bound motor protein myosin II.
6. During our microscopic examinations, we demonstrated that mitochondria movement in B-lymphoma cells occurs with the assistance of two motor proteins, kinesin and myosin VI. We were the first to establish a connection between the microtubule network and the function of B-lymphoma NTs. To our knowledge, we are the first to have conducted extensive investigations into potential motor proteins that regulate mitochondrial transport within nanotubes. We also found that the movement of mitochondria in NTs is not continuous but is characterised by alternating active (moving) and inactive (stationary) phases. This mechanism is similar to the nature of mitochondrial transport in axons. We were the first to describe this parallelism.

7. We proved that both macrophages and B-lymphoma cells transport immune costimulatory (CD86) membrane proteins through NTs. However, the localisation of the molecule differs between the two cell types: in macrophages, its transport occurs inside the NTs, whereas in B-lymphoma cells, it occurs in the wall of the plasma membrane.

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LIST OF PUBLICATIONS

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Publications related to the PhD work:

Halász H., Ghadaksaz AR., Madarász T., Huber K., Harami G., Tóth EA., Osteikoetxea-Molnár A., Kovács M., Balogi Zs., Nyitrai M. Matkó J., Szabó-Meleg E.: Live cell superresolution-SIM imaging analysis of the intercellular transport of microvesicles and costimulatory proteins via nanotubes between immune cells, Methods Appl. Fluoresc. 6(4):045005, (2018), doi: 10.1088/2050-6120/aad57d. **IF: 2,940, Q1**

Halász H., Tárnai V., Matkó J., Nyitrai M., Szabó-Meleg E.: Cooperation of various cytoskeletal components orchestrates intercellular spread of mitochondria between B-Lymphoma cells through tunnelling nanotubes, Cells 13(7), 607, (2024), doi: 10.3390/cells13070607, **IF: 6,0, Q1**

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Posters and presentations related to the PhD work:

international conference presentations:

Halász H., Ghadaksaz AR., Madarász T., Huber K., Nyitrai M., Matkó J., Szabó-Meleg E.: Visualization of transport properties of membrane nanotubes with live cell laser-scanning confocal and superresolution (SIM) microscopes. (8th Regional Biophysics Conference, 2018, Slovenia, presenter: Sz-M.E)

Halász H., Tárnai V., Nyitrai M., Matkó J., Szabó-Meleg E.: Examination of transport processes via membrane nanotubes with superresolution microscopy techniques. (Medical Conference for PhD Students and Experts of Clinical Sciences 2021, Hungary, Pécs)

Halász H., Tárnai V., Madarász T., Nyitrai M., Matkó J., Szabó-Meleg E.: Microscopic examination of motoproteins in the mitochondrial transport via membrane nanotubes. (Second Symposium on Super-resolution and Advanced Fluorescence Microscopy and István Ábrahám Memorial Workshop, 2022, Hungary, Pécs)

international conference posters:

Matkó J., **Halász H.,** Ghadaksaz AR., Madarász T., Huber K., Osteikoetxea-Molnár A., Tóth EA., Nyitrai M., Szabó-Meleg E.: Investigation of growth and intercellular properties of membrane nanotubes connecting immune cells by LC-CLSM and superresolution (SIM) imaging. (Methods and applications in fluorescence conference, 2017, Belgium, presenter: M.J.)

Halász H., Tárnai V., Madarász T., Nyitrai M., Matkó J., Szabó-Meleg E.: Studying the role of the microtubules in the formation and function of the membrane nanotubes of B-lymphocytes. (Hungarian Molecular Life Sciences, 2022, Hungary, Eger)

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Halász H., Tárnai V., Nyitrai M., Matkó J., Szabó-Meleg E.: Microtubules orchestrate the mitochondria transport via tunnelling nanotubes between B-lymphoma cells. (EMBL Symposium: Microtubules: from atoms to complex systems, 2024, Heidelberg, Germany)

Halász H., Tárnai V., Matkó J., Nyitrai M., Szabó-Meleg E.: Intercellular routes: membrane nanotube networks among B lymphocytes - essential drivers of growth and transport functions. (EMBL Symposium: Microtubules: from atoms to complex systems, 2024, Heidelberg, Németország, bemutatta: Sz.-M.E.)

national conference presentations:

Halász H., Ghadaksaz, AR., Huber K., Madarász T., Nyitrai M., Matkó J., Szabó-Meleg E.: Membrán nanocsövek transzportban betöltött szerepének tanulmányozása immunsejteken. (PEME XV. PhD konferencia, 2017, Budapest)

Halász H., Ghadaksaz, AR., Huber K., Madarász T., Nyitrai M., Matkó J., Szabó-Meleg E.: Immunsejtek közötti membrán nanocsövek – új „segéderők" az immunválasz hatékonyságának növelésében? (Fiatalok Európában konferencia, 2017, Pécs)

Ghadaksaz AR., Madarász T., **Halász H.,** Osteikoetxea-Molnár A., Tóth EA., Huber K., Nyitrai M., Matkó J., Szabó-Meleg E.: Immunsejtek közötti transzportfolyamatok vizualizálása. (48. Membrán-transzport konferencia, 2018, Sümeg, presenter: G.AR.)

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Halász H., Tárnai V., Madarász T., Nyitrai M., Matkó J., Szabó-Meleg E.: Membrán nanocsövek és a sejtváz fehérjék szerepe a mitokondriális transzportban. (XXV. Tavaszi szél konferencia, 2022, Pécs)

Halász H., Tárnai V., Nyitrai M., Matkó J., Szabó-Meleg E.: Citoszkeletális elemek együttműködése a mitokondriumok membrán nanocsöveken keresztüli transzportjában. (Magyar Mikroszkópiai társaság éves konferenciája, 2024, Siófok)

national conference posters:

Halász H., Ghadaksaz AR., Huber K., Madarász T., Nyitrai M., Matkó J., Szabó-Meleg E.: Membrán nanocsövek-Egy új útvonal az immunsejtek kommunikációjában. (videoposter, Magyar Mikroszkópiai társaság éves konferenciája, 2018, Siófok)

Halász H., Ghadaksaz, AR., Madarász T., Nyitrai M., Matkó J., Szabó-Meleg E.: Membrán nanocsövek transzport tulajdonságainak vizsgálata szuperrezolúciós mikroszkópiával. (poster, XXVII. MBFT kongresszus, 2019, Debrecen)

Halász H., Tárnai V., Matkó J., Nyitrai M., Szabó-Meleg E.:Citoszkeletális elemek kooperációja B sejtek közötti membrán nanocsöveken keresztüli mitokondrium transzport szabályozásában. (poster, 53. Membrán Transzport konferencia, 2024, Sümeg, presenter:T.V.)

List of presentations and posters not related to the dissertation:

Hencz, AJ., Nyitrai M., Bugyi B., Madarász T., **Halász H.,** Türmer K., Peter G., Matkó, J., Szabó-Meleg E.: TR100 hatása a membrán nanocsőhálózatokra. (poster, XXV. MBFT kongresszus, 2017, Szeged, presenter: H.AJ.)

Madarász T., **Halász H.,** Brunner B., Bisi S., Scitall G., Nyitrai M., Szabó-Meleg E.: I-BAR and IRSp53 proteins affect actin polymerization and membrane nanotube formation. (poster, 44th FEBS Congress, 2019, Poland, Krakow, presenter: SZ-M.E.)

Halász H., Madarász T., Nyitrai M., Matkó J., Szabó-Meleg E.: Fundamental growth determinants and transport functions of B cell membrane nanotubes (2022, Second Symposium on Super-resolution and Advanced Fluorescence Microscopy and István Ábrahám Memorial Workshop, Pécs, presenter:Sz-M.E.)

Madarász T., **Halász H.,** Szeiliné Türmer K., Matkó J., Nyitrai M., Szabó-Meleg E.: How membrane sculpturing proteins influence the growth and morphology of membrane nanotubes? (2022, Second Symposium on Super-resolution and Advanced Fluorescence Microscopy and István Ábrahám Memorial Workshop, Pécs, presenter: M.T.)