FINAL PROJECT CLOSING REPORT: "*Membrane Nanotube Networks between Immunocytes: regulating factors and functional significance in cell-to-cell communication with special attention to B and T cells*" (K104971 research project). *2017-10-30*

The research project based on the following major scientific questions:

1.) What conditions are optimal for B cells – key cellular components of the humoral immune response – to grow nanotubes and what are the basic properties of these nanotube bridges, compared to other already known nanotubular structures bridging various plant and animal cell types? ***2.)*** What is the role of the F-actin and microtubule cytoskeletal components in growth/retraction of nanotubes? Are motor proteins (e.g. myosins) involved in regulation of nanotube (NT) growth? ***3.)*** Does intracellular Ca^{2+} level and cell surface integrins play any regulatory role in NT-growth? ***4.)*** Does the actual membrane lipid composition have any impact on the growth and mechanical properties of NTs? ***5.)*** Can these lymphocytes communicate with each other via intercellular transport of immunoregulatory objects and what might be its functional significance? What kind of immunoregulatory molecules or other larger structures (vesicles, organelles) are transported and by what mechanism between B cells (homotypic NT network) or between B and T lymphocytes (heterotypic NT-network)? ***6.)*** Can these lymphoid NT-networks form in 3D gel matrices? Do such lymphoid NT bridges form *in vivo*, e.g. in lymphoid organs?

In the first period, the participants optimized the environmental conditions for simulating closely physiological B cell environment (this became later as 'standard live cell imaging condition' for comparative studies) and compared various immature and mature B cells in terms of their NT-growing properties. The standard, optimized conditions for imaging nanotube networks with specialized microscopic techniques, such as TIRF, AFM and super-resolution structured illumination (SR-SIM) microscopy, were also set at our collaboration partners, at Semmelweis University of Budapest, Department of Biophysics and Radiation Biology, as well as at University of Pécs, Medical Faculty, Department of Biophysics. The methodologies for cell labeling and quantitative evaluation of NT images were also worked out in this period and a new microscopic technique, Differential Polarization Laser Scanning Confocal Microscopy (DP-LSM) was set up on our Olympus CLSM system to study membrane lipid ordering, in collaboration of Drs. Gabor Steinbach and Győző Garab (BRC of HAS, Szeged). The basic characterization of the B cell nanotubes – related to questions ***1.-3.*** - was finished by the end of the second period, in full accordance with the research plan.

In the third period there was only one significant small change in the project lining as compared to the original research plan, namely, based on preliminary trials and calculations, we gave up proteomic analysis of isolated NTs. The main reasons were the extremely time-consuming NT-isolation and the high amount of NT-proteins requested for a reliable proteomic analysis. Instead, as we noted in the third period closing report, we decided to make a comparative, full scale, GC-MS based lipidomic analysis (this analysis requires only small amount of cell membrane isolate and more rapid than the proteomic analysis). The results of such analysis are expected to help us to understand the way of contribution of membrane lipid species to growing of nanotubes (question ***4.***). This full scale lipidomic analysis was successfully done (for detailed results see next paragraphs).

In the fourth period several notable changes happened with the participants. Eszter Angéla Lomen-Tóth left for maternity leave and a little later Anikó Osteikoetxea-Molnár left to England (married). Thus, two basic participant researchers fell out from the project unexpectedly enough, in the middle of a busy period of the project. A replacement researcher (Krisztina Huber) also left after 4 months, (for better salary), before the end of the project, so

the PI should have finished the project with the assistance of an MSc student (Mr. AliReza Ghadaksaz) and a part-time postdoc (Dr. Krisztián Papp, for three months). Mostly due to this situation, the lymph node imaging subproject could not be fully finished by the end of the project. The other important, originally planned investigations on intercellular transport of immunomodulatory components (intracellular microvescles, mitochondria or costimulatory CD86 membrane proteins), on 3D and heterocellular (B-T cell) nanotubular bridges were finished successfully and prepared or already submitted for publication, respectively.

In summary, more than 80% of the original questions were sufficiently worked out and answered by the results of the project (for details see the next session) and these results and the underlying methodical developments were already published in **4 peer reviewed journal articles**. A **5th research article** was also submitted (10-30-2017, to Methods and Applications in Fluorescence - IOP Science) and another one is currently under preparation.

Based on the project results and its international reference environment, **2 BSc** (Ádám Oszvald, 2014; Péter Revisnyei, 2017) and **2 MSc** (Ádám Oszvald, 2016; AliReza Ghadaksaz, 2017) students were involved in the research work and their diploma dissertation were successfully defended, as well as defenses of **2 PhD dissertations** (Anikó Osteikoetxea-Molnár, 2017, Eszter Angéla Lomen-Tóth, 2018) are also in progress at the Faculty of Science, Eötvös Loránd University, Budapest.

MAJOR RESULTS OF THE PROJECT:

1.) Characteristic properties of the yet unknown B cell nanotubes.

- B cells spontaneously form nanotubes, but *only at physiological conditions (37^0C, 5% CO_2)* and *on extracellular matrix (ECM) protein coat* (e.g. fibronectin or laminin). Our results also pointed on an important fact that in contrast to mature B cells, immature or pre-B cells do not form NTs, even at such optimal conditions. Selective expression of certain *integrin α and β chain combinations, as specific receptors of the ECM proteins* (e.g. α5/β1 or α6/β1 for fibronectin), was found as *one of the key factors controlling cell adhesion/spreading, a prerequisite of nanotube growing*. Thus, we can conclude that *B cells can spontaneously form NTs in vitro, but NT formation is maturation-, differentiation stage-dependent and requires integrin-ECM contact.*
- High resolution microscopic (AFM, SEM, SR-SIM) and fluorescence CLSM analysis demonstrated that most of the *NTs connecting B cells contain both F-actin bundles and microtubules, relatively thick, can be branched and show tunneling nanotube (TNT) morphology*. *F-actin proved to be the active skeleton protein* of these TNTs, while degradation of microtubules did not affect TNT growth frequency.

2.) Cytoplasmic Ca^{2+} level, F-actin and myosin 2A all control TNT growth or retraction in B cells

- *Ca^{2+}-induced*, cofillin- and gelsolin-mediated dynamic *changes in the F-actin polymerization-depolymerization equilibrium may control in B lymphocytes growth or retraction of NTs.*
- Inhibition of myosin 2A (an abundant motor protein in B cells) directly or the activity of ROCK enzyme regulating it, both resulted in a highly increased extent of NT formation, suggesting that *myosin 2A is a negative regulator of NT formation and its activity in*

lymphocytes can shift the balance of cellular machineries controlling cell protrusion or contraction towards contraction.

3.) Intercellular transport of organelles, molecules and vesicles with potential immuno-regulatory function takes place via nanotubes between B cells.

- The good lateral resolution (d: 80-90 nm) of SR-SIM imaging allowed us to detect *transport of ganglioside-rich microvesicles between connected B cells via TNTs, in real time*. Immunocytochemical post-labeling showed that *part of these microvesicles contain also MHC-II or CD86 costimulator proteins*, but traffic of *these membrane proteins by diffusion in the membrane of the tubes* was also observed. Such *homo-typic intercellular exchange* of these molecules may contribute to the process of "*antigen-spreading/cross-dressing*" that may *positively influence T cell activation and therefore the intensity of cellular immune response.*

- Using fluorescent markers and live cell imaging confocal or SR-SIM microscopies we could also detect *intercellular transport of lysosomes and mitochondria between TNT-bridged B cells*. Note that mitochondria were transported mostly from healthy intact cells towards cells of dying/apoptotic morphology. These observations allow us to conclude that *both antigen-presenting capacity and homeostasis of B cell populations may be influenced by intercellular exchange of lysosomes and mitochondria, respectively*.

4.) Lipid modifications of the B cell plasma membrane results in altered nanotube growing capacity and mechanical properties.

- First we set up two different methodical approaches for fluorescent labeling of lymphocytes and for quantitative detection of lipid molecular ordering in their plasma membrane. A *differential polarization laser scanning confocal microscopic setup (DP-LSM) was developed* on our Olympus FluoView CLSM workstation in collaboration with Dr. Győző Garab's group (BRC of HAS, Szeged) and *successfully applied to test changes of lipid order in B cells upon membrane modifications.*

- *Fluidization of the plasma membrane* (with PUFAs linoleic acid or docosahexanoic acid) *results in higher NT growth frequency, lower membrane orderedness and smaller "keeping force" in AFM mechanomanipulation*. In contrast, *crosslinking of membrane gangliosides results in lower NT number, higher order and keeping force*, suggesting that *an optimal membrane fluidity/order is essential for NT-growth*.

- *Depletion of membrane cholesterol, sphingomyelin* from B cells or a mutation-derived *general sphingolipid deficiency* in HeLa cells all *resulted in largely decreased NT-frequency, suggesting a critical role of these lipids in NT-growing*.

5.) Membrane lipid composition of immature and mature B cells is significantly different in lipid species influencing nanotube growth as shown by a full scale GC-MS lipidomic analysis.

- To understand the striking difference in NT-growing capacity of immature and mature B cells, a comparative, correlative assay of 9 mouse and human B cell lines with different maturation/differentiation stage was performed in terms of their membrane cholesterol and ganglioside levels and NT-frequency. A *linear correlation was found between NT-*

growth frequency and membrane ganglioside (GM_1/GM_3) level, while NT-growth has shown a maximum with increasing membrane cholesterol level. These results suggest that the *actual number of intact lipid rafts domains is a key factor in NT-growth possibly via coupling the membrane to F-actin, while the too high cholesterol level may decrease membrane elasticity, which is unfavorable for NT-growth*.

- A comparative full scale lipidomic analysis on immature and mature B cells incapable or capable to grow nanotubes, respectively, strongly supported the *favorizing role of GM_3 gangliosides and other raftophilic lipids with short fatty acyl chains in NT-growth*, in accordance with the result shown above. In addition, the much higher level GlcCeramide, LacCer and the DHA-containing inner lipids in mature B cells is also consistent with this picture. All these results suggest that the *plasma membrane lipids may promote nanotube-growth through the following factors: high raft level, favorable membrane curvature, favorable elasticity and high density of membrane-F-actin couplings*.

6.) Mechanism of the intercellular transport of ganglioside (raft)-enriched microvesicles and costimulatory membrane protein CD86 between B and T lymphocytes and macrophages.

Since extracellular microvesicles (EVs) were shown to have serious immuno-modulatory (both activatory or inhibitory) potential (Thery C et al, 2009. Nat Rev Immunol, 9: 581-593) we were interested next how the novel intercellular transfer pathways of intracellular microvesicles (IVs) and other immunoregulatory proteins described here work between B and T cells or macrophages.

- After challenging B cells with fluorescent cholera toxin B, following rapid binding a subsequent internalization and *formation of fluorescent (ganglioside-rich) microvesicles (100-1000 nm diameter size range) was found, saturating in ca. 30 minutes*. These *microvesicles showed then a slow, two-way traffic between the TNT-bridged B cells*.

- In an inhibition analysis we found that *both destruction of F-actin network (with latrunculin B) or inhibition of the abundant myosin 2A motor protein (with p-nitro-blebbistatin) largely decreased the number of mobile vesicles in the TNTs*. In contrast, inhibition of the microtubule-dependent dynein, another abundant motor protein in B cells, with ciliobrevin D did not affect the transport of these microvesicles. Thus, we conclude that *intercellular transport of these potentially immunomodulatory microvesicles via nanotubes is mediated by the acto-myosin system, rather than microtubule-dependent motor proteins*.

- Since the membrane level of CD80 or CD86 costimulatory proteins in antigen presenting cells (APC) sensitively influences their T-cell activatory potential, next we investigated in B cells and macrophages (two APCs) whether these molecules are transported between these cell types via TNTs. First, after optimizing the transfection protocols, *successfully transfected A20 B cells and Raw 264.7 macrophages with GFP-CD86*. Looking at intercellular transport of this fluorescent construct with live cell SR-SIM imaging, interestingly we found that *in B cells the transport took place dominantly by diffusion in the membrane of TNTs, while in macrophages GFP-CD86 was transported in large microvesicles, in concentrated fashion*. Note that *CD86 was also transferred from B cells to T cells through the membrane of TNTs connecting these cells*. These data suggest that *CD86, an import costimulatory protein can be exchanged between B cells and macrophages, alike, although by a cell-specific traffic pathway*. This in turn may increase

the mathematical chance of efficient T cell activation within a B cell or macrophage population.

➢ We could detect heterocellular nanotubes (HC-NT) between 2PK3 antigen presenting B cells (loaded with influenza virus HA peptide) and a cognate HA-specific T cell line (IP12-7), in co-culture (Figure 1. g-j). Analyzing time-dependence of HC-NT formation (panel **d**) and synapse formation (panel **f**) between these cells, we found very similar time profile, suggesting that formation of NTs correlates somehow with immunological synapse formation.

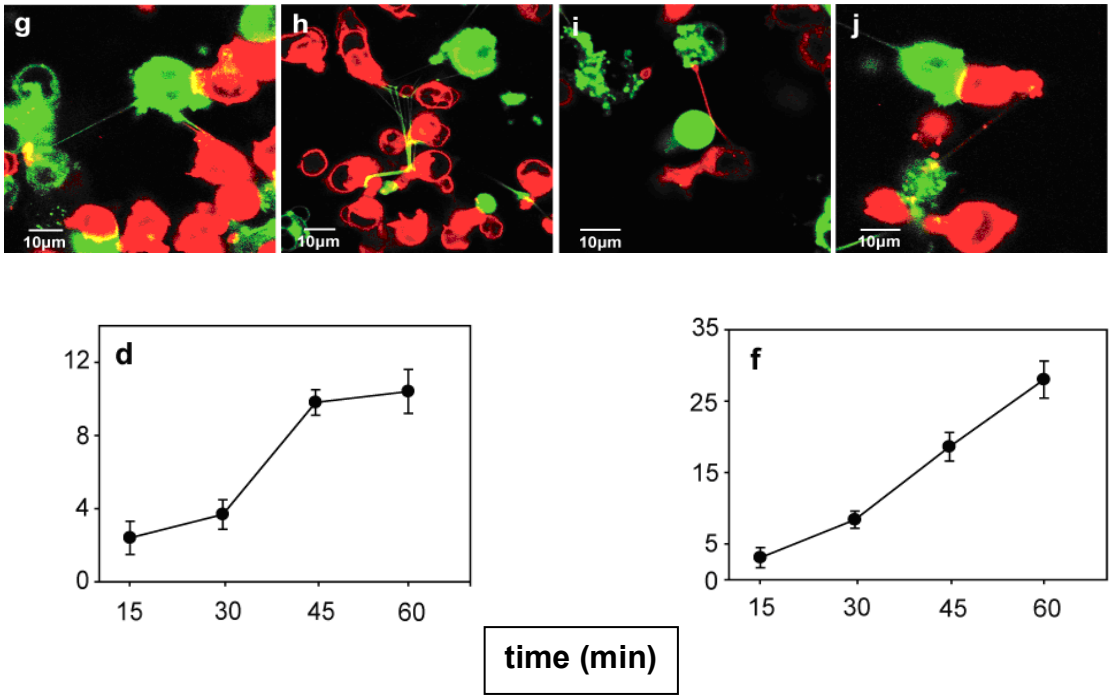

Figure 1: Panels g-j show representative live cell CLSM images of DiO (green) and DiI (red) labeled 2PK3 B cells and IP12-7 T cells, respectively. Notice the long and thin NTs with different color, suggesting that both cells try to communicate with the other one. Frequency (%) of heterocellular NT formation (**d**) and the frequency of immunological synapse formation (**f**) as a function of time in co-culture is shown here as a mean of three independent experiments together with SD.

➢ Concerning imaging the lymph nodes with 2 photon microscopy - in order to detect NTs between T cells - we started the experiments with isolating LNs from mice immunized with KLH antigen. Using adaptive transfer of green cell tracker labeled KLH-specific T cells into the mice after a few days the LNs were isolated and investigated in 2P microscope. We faced several technical problems to be solved. First the cell density and their autofluorescence in the green fluorescence spectral region was extremely high. At the same time the number of T cells accumulated after immunization into the LN was low. At this point, to continue this line, we decided to change the injection strategy (from tail vein to eye) and apply Red cell tracker for T cells, in order to avoid interference with the high autofluorescence. However, in the lack of time and researchers this experiment could not be finished.

➢ During the project two fruitful and long term collaborations formed between the PI lab and two other research sites (Semmelweis University, Department of Biophysics and Radiation Biology; University of Pécs, Department of Biophysics). Here several open subprojects

remained to finish, such as e.g. the above mentioned 2PM in vivo imaging or the role of I-Bar proteins (IRSp53 in B cells) in formation of nanotubes (see Abstracts in publication list).

In extenso publications from the results of the project:

1.) Steinbach G, Pawlak, K, Tóth EA, Molnár A, Matkó J, Garab G: Mapping microscopic order in plant and mammalian cells and tissues: Novel differential Polarization attachment for new generation confocal microscopes (DP-LSM). ***METHODS AND APPLICATIONS IN FLUORESCENCE-IOP Science*** **(2014)** Vol. 2, No. 1, 015005 (IF: 2.429)

2.) Herbáth M, Papp K, Balogh A, Matkó J, Prechl J: Exploiting fluorescence for multiplex immunoassays on protein microarrays. ***METHODS AND APPLICATIONS IN FLUORESCENCE-IOP Science*** **(2014)** Vol. 2, No. 1, 032001 (IF: 2.429)

3.) Osteikoetxea-Molnár A, Szabó-Meleg E, Tóth EA, Oszvald Á, Izsépi E, Kremlitzka M, Biri B, Nyitray L, Bozó T, Németh P, Kellermayer M, Nyitrai M, Matkó J: The growth determinants and transport properties of tunneling nanotube networks between B lymphocytes. ***CELL MOL LIFE SCIENCES (2016)*** 73:4531-4545. (IF: 5.788)

4.) Tóth EA, Oszvald Á, Péter M, Balogh G, Osteikoetxea-Molnár A, Bozó T, Szabó-Meleg E, Nyitrai M, Derényi I, Kellermayer M, Yamaji T, Hanada K, Vígh L, Matkó J: Nanotubes connecting B lymphocytes: High impact of differentiation-dependent lipid composition on their growth and mechanics. ***BBA-MOLECULAR AND CELL BIOLOGY OF LIPIDS (2017)*** 1862: 991-1000. (IF: 5.547)

5.) Matkó J, Ghadaksaz AR, Halász H, Madarász T, Harami G, Huber K, Tóth EA, Osteikoetxea-Molnár A, Kovács M, Nyitrai M, Szabó-Meleg, E: Some mechanistic details of intercellular transport of microvesicles and costimulator proteins via nanotubes between immune cells are revealed by live cell SR-SIM imaging. ***METHODS AND APPLICATIONS IN FLUORESCENCE-IOP Science (2017***, ***October 30***) submitted (IF: 2.656)