Live cell confocal fluorescence microscopic (LC-CLSM) imaging of tunelling nanotube networks (TNTs) between B lymphocytes and the neutrophil extracellular traps (NETs)*

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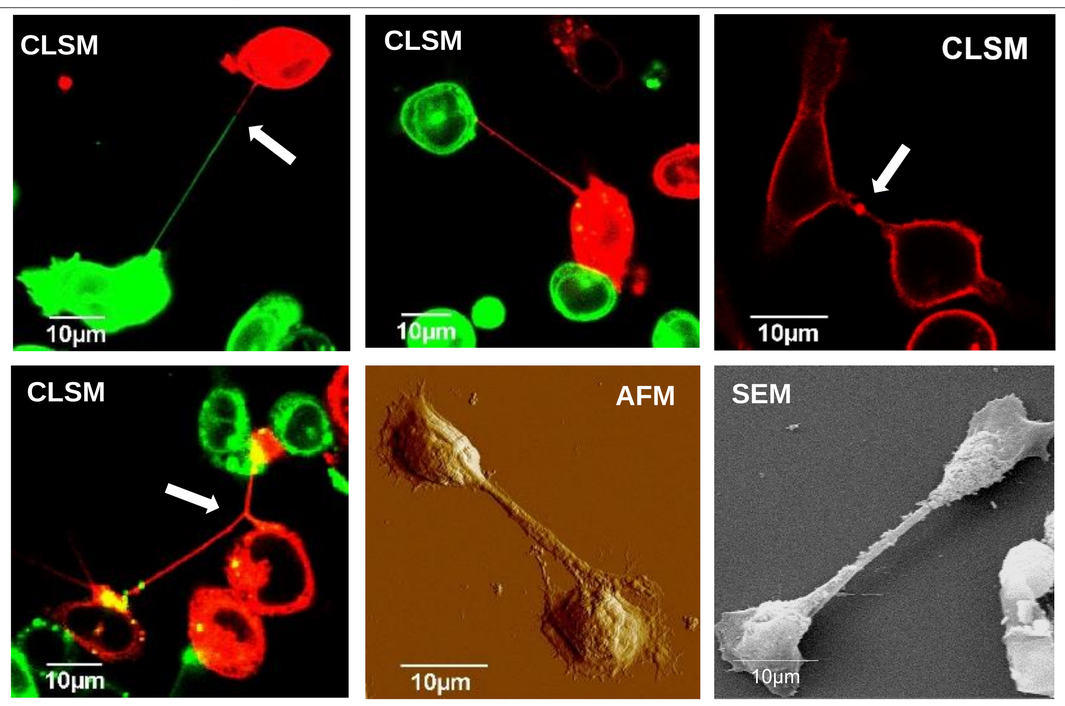
The aim of the MedInProt 2014 project was to set up a flexible, temperature controlled gas incubation system to our confocal laser scanning fluorescence microscope (CLSM) in order to be able to monitor various special processes between cells of the immune system and/or the complement system, such as intercellular communication of lymphocytes through tunelling membrane nanotubes (TNTs) or formation of neutrophil extracellular traps (NETs) and their regulation by the complement factors under live cell conditions.

The system: *Ibidi (Germany)* can receive different formats of sample holders, such as borosilicate bottomed microplate chambers or Petri dishes, controls the temperature in the holder with ±0,1 °C precision and provides controlled gas atmosphere (e.g. 5% CO_2 or hypoxic conditions) at controlled humidity. The system was attached to an Olympus FluoView 500, inverted IX81 microscope based CLSM workstation (see figure).
Nanotube and NET formation was monitored and analysed with the above Olympus CLSM system using 60x oil immersion objective (N.A.:1.1) or occasionally Structured Illumination Microscopy (SIM) (Zeiss Elyra S1) at PTE Biophysics Dept. Pécs. Statistical analysis: the occurance frequencies were calculated from 200-400 cells/sample and analyzed by SigmaPlot software.

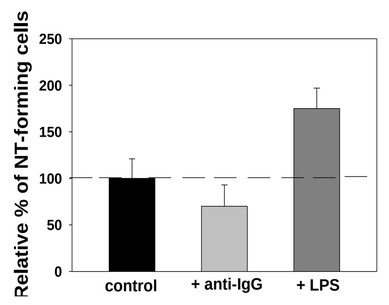
RESULTS:

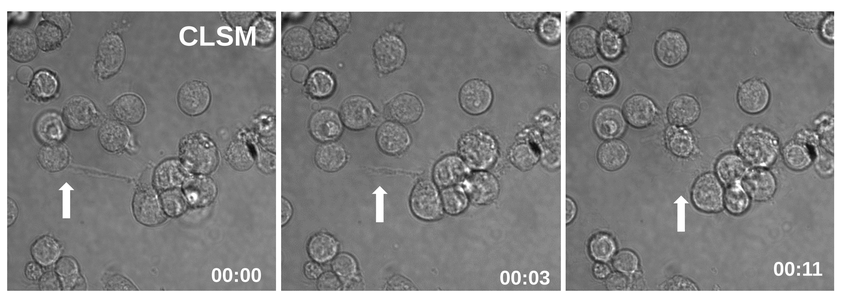
1. We have shown that 10-15% of mature murine B cells spontaneously form long and relatively wide membrane nanotubes with different mechanisms (e.g. following cell division), under live cell conditions (fibronectin coat, 37°C, 5%CO_2). NT growth was critically depended on the fibronectin-α5β1 integrin interaction. We could first detect two way transport of membraneous microvesicles between adjacent B cells through the nanotubes, which in antigen-presenting B cells may represent a novel immunoregulatory pathway.

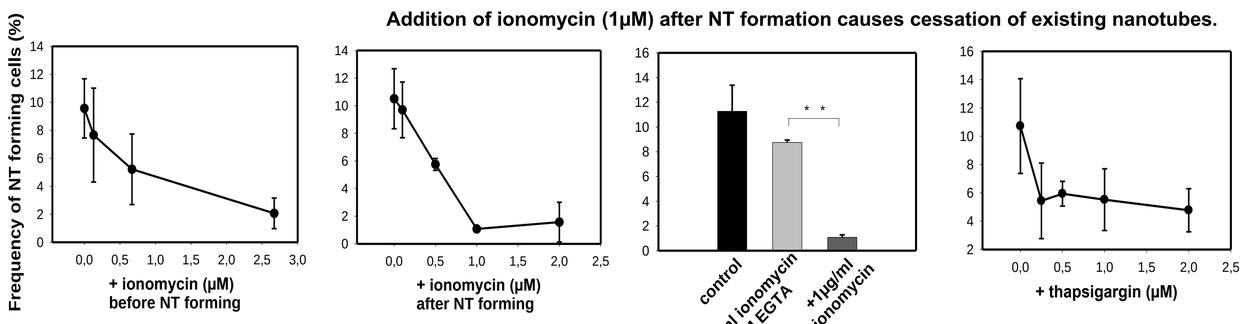


Cells were stained with DiI/DiO dyes for live cell CLSM imaging.

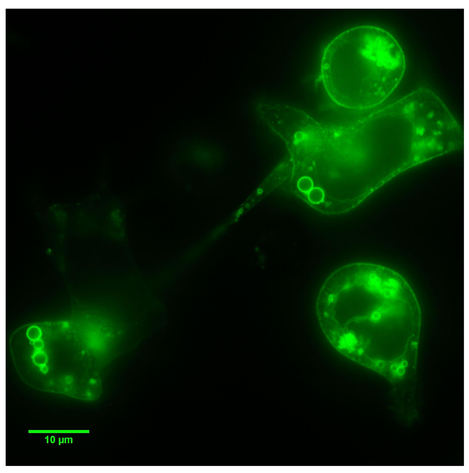
2. Local cytoplasmic free Ca^{2+} level controls the formation-collapse equilibrium of B cell nanotubes



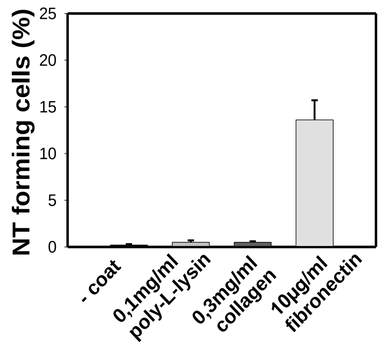


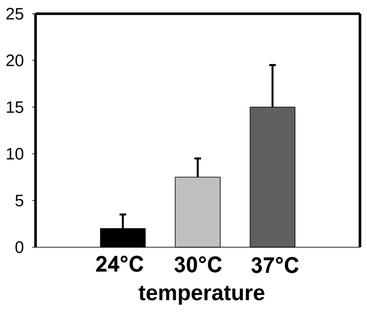


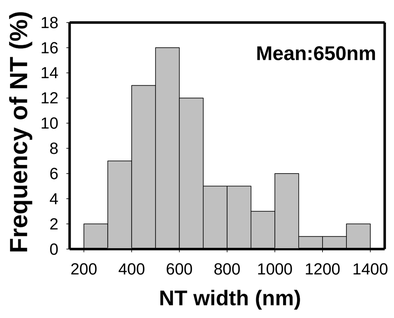
3. Visualization of neutrophil extracellular traps

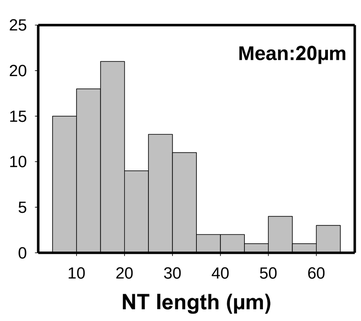


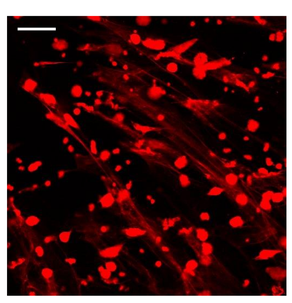
SIM image (ca. 80-90 nm resolution) of Alexa488-choleratoxin-B labeled A20 B cells. The small membraneous microvesicles transported through the NTs are clearly visible



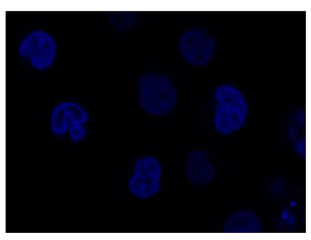


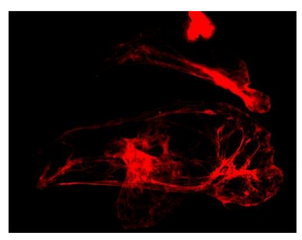






Human neutrophil granulocytes isolated from peripheral blood release extracellular traps (NETs) upon stimulation with 100 nM PMA for 3h, a mesh of DNA and associated proteins. Staining with Sytox Orange. Scale bar, 100 μm





PLB-985 cells were differentiated in the neutrophil direction by dimethyl-formamide treatment. Draq5-staining shows the nuclei of the cells (blue). Upon treatment with 100 nM PMA for 3h, NETs are visualized by staining the extracellular DNA with Sytox Orange (red).

SUMMARY:

- We have shown that under closely physiological conditions about 10-15% of B cells spontaneously form membrane nanotubes (5-65 μm long and 200-1400 nm wide) connecting two or sometimes multiple B cells that can be differentially modulated by various stimuli of B cells.
- The nanotubes contain F-actin, the integrity of which seems essential in nanotube growth and also microtubules that might be important in transport processes of membrane vesicles across the TNTs.
- The cytoplasmic Ca^{2+} level of B cells also has high impact on the growth vs. retraction equilibrium of nanotubes, presumably through activating actin-regulatory proteins cofilin and gelsolin. The exact connection between their regulation roles in nanotube formation of lymphocytes still remained open question. Thus, further investigations are running currently in our lab.
- Neutrophil extracellular traps can be visualized and activation events, such as change in cytoplasmic Ca^{2+} level and cell spreading, can be followed. Our results show that complement factor H can inhibit PMA-induced NET formation. Further studies regarding the mechanism of this effect are underway.

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