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Introduction and the aims of STSM. NLRP3 inflammasome is a multiprotein complex mediating inflammatory responses in a variety of common diseases, such as gout, metabolic diseases and Alzheimer's disease. Upon NF-κB activation and stimulation with a plethora of diverse triggers leading to intracellular Ca^{2+} mobilization and K^{+} efflux, NLRP3 oligomerizes. NLRP3 oligomerization induces polymerization of adaptor ASC, which enables polymerization and self-activation of caspase-1, further activating proinflammatory cytokines IL-1β and IL-18 (Figure 1). The molecular mechanism of NLRP3 oligomerization remains largely unknown.
In order to define the roles of selected domains of NLRP3 in NLRP3 inflammasome assembly 20 truncated NLRP3 mutants were expressed in NLRP3-deficient macrophages yielding proteins comprising PYD and NBD domains with C-terminal segments of different length. We showed that mutants lacking LRR domain are fully responsive to various soluble and particulate instigators.
The main aim of the proposed STSM was to define structural determinants of NLRP3 in response to various physiological processes triggering NLRP3 inflammasome assembly. Particularly, we followed inflammasome activation upon perturbed ion homeostasis mediated by P2X7, which has not been followed by the researcher in the home laboratory. The second aim was to receive additional information about NLRP3 conformation/ oligomerization upon trigger addition.

Methods. ATP- and nigericin-mediated activation of macrophages expressing selected NLRP3 variants was followed with immunological and biochemical techniques (Figure 1). Endogenous ASC speck formation was followed by immunocytochemistry and fluorescence microscopy. Caspase-1 activation was followed by western blot and IL-1β processing by western blot and ELISA. Pyroptosis was determined by LDH release. Conformational change/interaction between NLRP3 molecules was followed by BRET assay.

Results. Previously we have shown that d8 NLRP3 variant, which is lacking LRR domain responds as full-length NLRP3 to triggers nigericin, imiquimod, silica nad alum, whereas 21-amino acid residues shorter variant (d7) did not respond to any of triggers. During STSM we have shown that the same response was observed also to P2X7-dependent ATP stimulation. While inflammasome activation in the home laboratory was mostly followed by IL-1β maturation, the findings were during STSM confirmed by other functional readouts, such as LDH release and caspase-1 activation (Figure 2). Additionally, we have shown that d7 mutant is unable to support ASC speck formation, which is upstream of caspase-1 activation (Figure 2C). Further, we followed conformational changes and interactions in various setups of bioluminescence resonance energy transfer (BRET) measurement. Intramolecular BRET results provided information on the distance between luciferase and YFP, showing that these tags are closer in the full-length protein than in d8, which is confirming the homology models based on the structure of similar protein NLRC4 (Figure 3 A,B). Surprisingly, the BRET in d7 is much lower (Fig. 3C), suggesting that the conformation of d7 is different than conformation of d8, despite similar length. We were also able to show that nigericin is able to induce conformational change in full length, d8, and also in d7 NLRP3, suggesting that d7 variant is able to respond to trigger, but unable to support inflammasome assembly. The majority of BRET experiments were performed in HEK293 cells upon transient transfections, but were confirmed in stably transduced immortalized bone marrow-derived macrophages. We have also shown that YFP and luciferase-tagging of wild-type or d8 mutant did not interfere with inflammasome activation. Further, intermolecular BRET, where one NLRP3 molecule is tagged with YFP and the other with luciferase, was followed. Different combinations of full length and truncated variants, tagged either at N- or C-termini were used. We observed increase in BRET upon nigericin treatment only for selected combinations of molecules, which is very interesting and will be the subject of further research.
I believe that STSM was successfully accomplished. I gained new knowledge and experience particularly on BRET measurement and plenty of results (the majority of experiments were repeated at least three times) which will be used in the joint publication. Selected results are presented in Figs. 2 and 3 on the following pages.
Thank you very much and best regards,

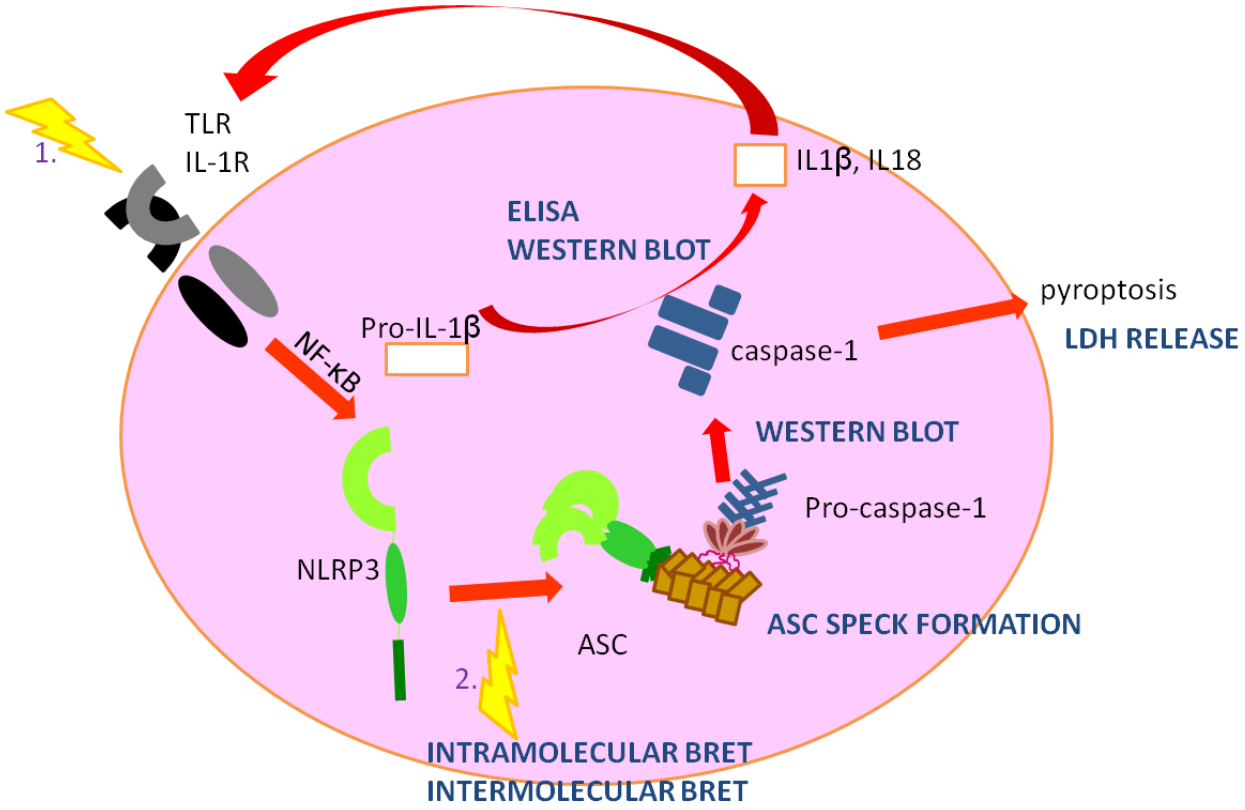

Figure 1: Schematic representation of NLRP3 inflammasome activation pathway with methods used during STSM. NLRP3 inflammasome is a multiprotein complex composed of receptor NLRP3, adaptor ASC and pro-caspase-1.There are two signals needed for the NLRP3-inflammasome activation, the first one, also called the priming signal leads to induction of NF-κB and expression of pro-IL1β and NLRP3 receptor. The second signal triggers the assembly of NLRP3 inflammasome. First NLRP3 changes conformation and/or oligomerizes, than recruits adapter ASC, which polymerizes and recruits pro-caspase-1. Procaspase-1 self-activates and cleaves pro-IL1β.

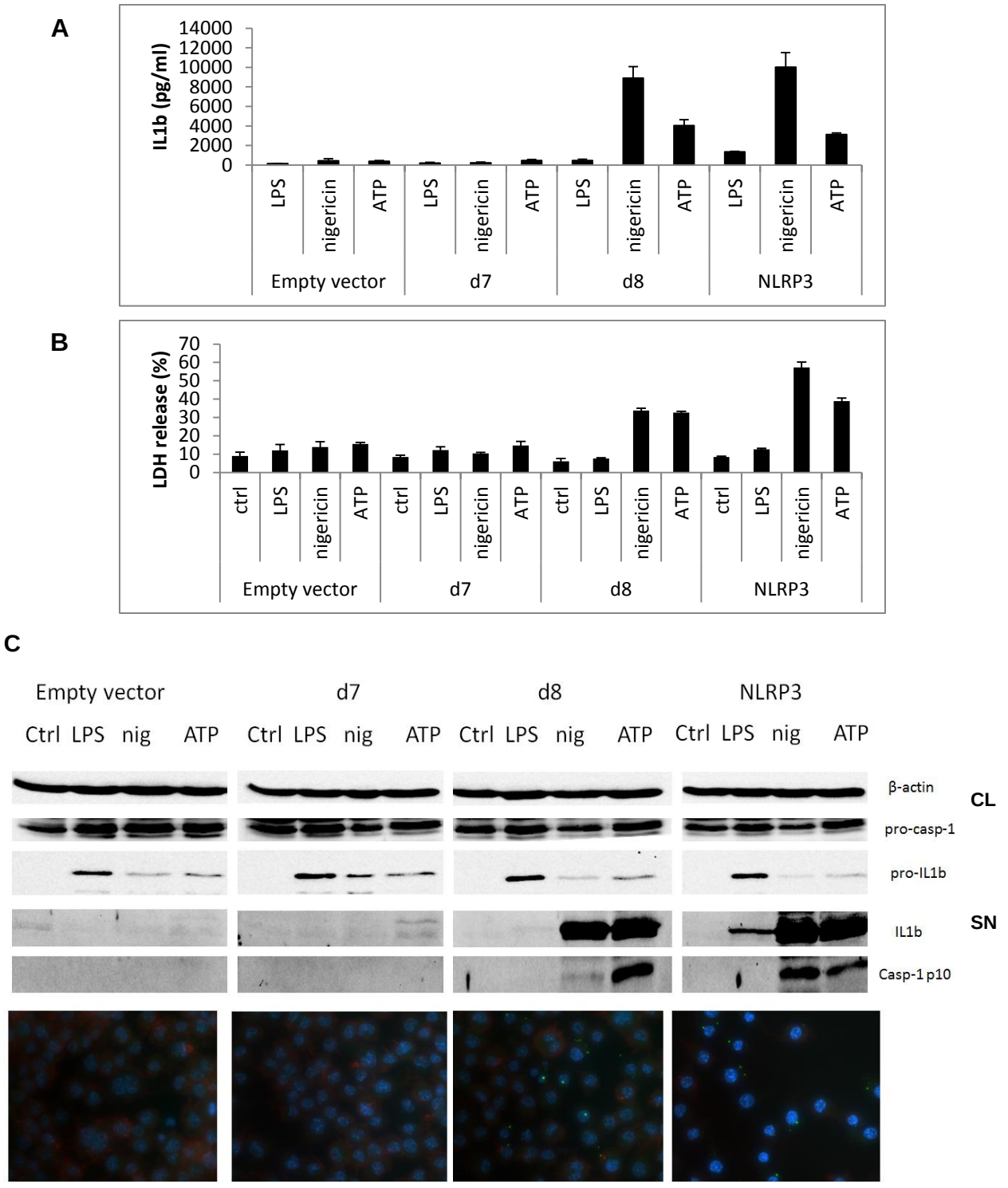

Figure 2: NLRP3 activation of different human NLRP3 variants was followed at various stages of NLRP3 inflammasome activation. Downstream IL-1β processing was followed by ELISA (A) and Western blot (C). Caspase-1 activation was followed by western blot (C). Pyroptosis, a caspase-1 dependent cell death, was followed by LDH release (B). C, bottom row: nigericin-treated cells were stained for ASC (green), actin (red) and nuclei (blue) and observed by fluorescence microscopy. ASC speck formation was only observed for d8 and full-length NLRP3 but not for nigericin treated d7 NLRP3 or empty vector transduced immortalized macrophages.

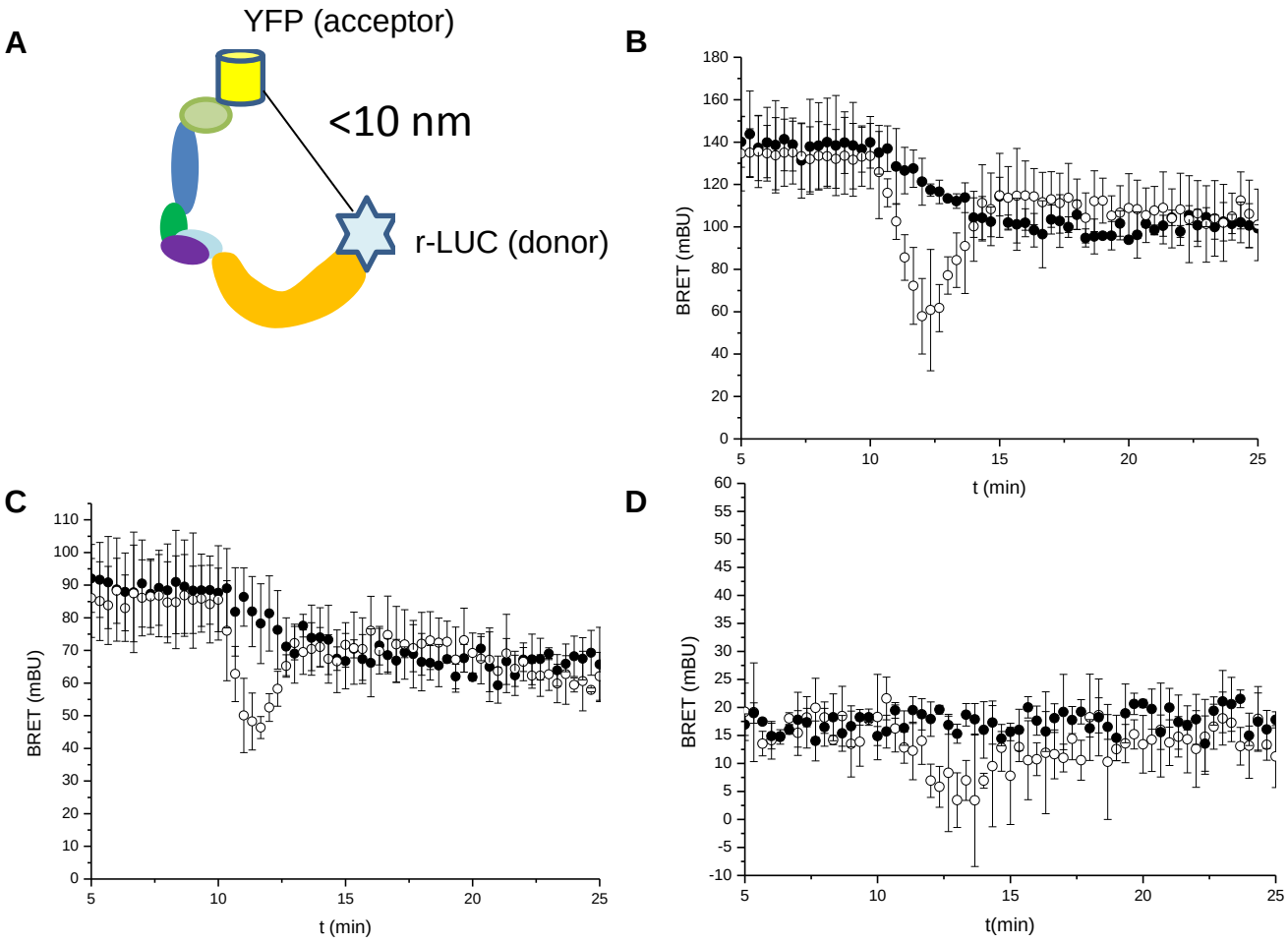

Figure 3: Intramolecular BRET was followed prior and after nigericin stimulation. BRET can be observed when the distance between the donor and acceptor does not exceed 10 nm (A). HEK293 cells were transfected with YFP NLRP3 LUC (B), YFP d8 LUC (C) and YFP d7 LUC (D) and their corresponding donor-only constructs. Luminescence at 528 nm and 485 nm was followed. 10 min after substrate coelenterazine H addition, either nigericin (empty circles) or buffer (full circles) was added and luminescence was followed for additional 15 minutes. BRET was calculated by the following formula: 1000*([LUM(528 nm)/LUM(485 nm)]$^{\text{YFP-NLRP3-LUC}}$ - [LUM(528 nm)/LUM(485 nm)]$^{\text{NLRP3-LUC}}$).