Supporting Information

Membrane Targeting enhances muramyl dipeptide binding to NOD2 and Arf6-GTPase in mammalian cells

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I. Experimental Procedures

Cell lines, reagents, and antibodies. Human embryonic kidney 293T (HEK293T) cell line was obtained from the American Type Culture Collection (ATCC). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher) with glucose, L-glutamine, and sodium pyruvate, which was supplemented with 10% final concentration fetal bovine serum (VWR) referred to as complete DMEM. Transient transfections were performed with Lipofectamine 2000 reagent diluted in Opti-MEM reduced serum media (Thermo Fisher). Luciferase reporter assays were performed with the Dual-Luciferase Reporter Assay System (Promega). Muramyl dipeptide (MDP), L18-MDP, and M-triDAP were purchased from Invivogen. GlcNAc-MDP and GlcNAc-MTP were synthesized as previously reported.[1] All SDS-PAGE was performed with Criterion TGX Stain-Free precast 4-20% gels with either 18 or 26 wells (Bio-Rad). All gels and western blots were imaged using ChemiDoc XRS System (Bio-Rad). Immunoprecipitations were performed with EZview Red Anti-HA affinity gel (Millipore Sigma). Anti-HA-peroxidase conjugated antibody (3F10) was purchased from Millipore Sigma (12013819001). Arf6-specific polyclonal antibody was purchased from Proteintech (20225-1-AP). Amersham ECL rabbit-IgG, HRP-linked whole antibody was purchased from Cytiva (NA934).

Plasmids and molecular biology. The plasmids pDSHA-Arf6-Myc, pCMV-HA-NOD2, PRDII-4x-luc, and pRL-TK were used as previously reported.[1,2] Site-directed mutagenesis was performed using the Q5 Site-Directed Mutagenesis Kit (New England Biolabs) using primers in Supplementary Table 1. Mutants not listed in the table were designed in a previous study.[2]

Plasmid	**Mutant**	Primers
pDSHA-Arf6-myc	N-term Swap	TTCGCCAACCTCTTCGGGAACAAGGAAATG GATGTTCCCCATGGTTCTATCTCCTTCTAAG
	K3R	AACCATGGGGCGGGTGCTATCCA CTATCTCCTTCTAAGCCTG
	S38I	GCTGGGCCAGATTGTGACCACCA TTCAACTTGTACAGGATTGTTG
	T44N	CACCATTCCCAATGTGGGTTTCAAC GTCACCGACTGGCCCAGC
	F47A	CACTGTGGGTGCCAACGTGGAGAC GGAATGGTGGTCACCGAC
	T53E	GGAGACGGTGGAGTACAAAAATGTCAAGTTC ACGTTGAAACCCACAGT
	K58S	CAAAAATGTCTCGTTCAACGTATGGGATGTG TAAGTCACCGTCTCCACG
	N60T	TGTCAAGTTCACCGTATGGGATG TTTTTGTAAGTCACCGTC
	W62A	GTTCAACGTAGCGGATGTGGGCG TTGACATTTTTGTAAGTCAC
	Y77A	CTGGCGGCATGCCTACACTGGGAC AGCGGCCGGATCTTGTCC
	TG79QN	GCATTACTACCAGAATACCCAAGGTCTCATCTTCGTAGTGGACTGCGC CGCCAGAGCGGCCGGATC
	CA90SN	CGTAGTGGACTCCAATGACCGCGACC AAGATGAGACCTTGGGTC
pCMV-HA-NOD2	C395S	TGAACGCCACTCCTCCCCGACCG CGATCCGTGAACCTGAACTTGAACTCG
	C1033S	CAAGCTCGGCTCCAGGGACACCA TCAACCTCCTCTAGAGAGAAAGTGTTC

Co-immunoprecipitation of HA-NOD2 and Arf6. 0.15 x 10^6 HEK293T cells per well were seeded for 16-20 hours in 12-well plates in complete DMEM. Cells were transfected the following day with 1.0 µg HA-NOD2 plasmid, 1.0 µg Arf6-myc plasmid, and 4.0 µL Lipofectamine 2000 per well. MDP or other NOD2 agonists were added 6 hours post-transfection and incubated overnight. Afterward, cells were harvested and lysed in 0.25

mL Brij Lysis buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 0.2% Brij 97) on ice. Lysates were cleared at 15,000 xg for 10 min at 4 °C. 10 µL of clarified lysate were set aside to be run on a separate SDS-PAGE gel. 200 µL of clarified lysate were gently mixed with 10 µL of anti-HA immunoprecipitation beads for 1 hour at 4 °C and then washed three times with 0.5 mL ice-cold RIPA buffer (50 mM HEPES pH7.4, 150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS). 10 µL of 4x Laemmli sample buffer was added to the beads and boiled for 5 min to elute protein. Samples were then run on SDS-PAGE gel. Stain-Free imaging was used to view total protein. The gel was then transferred (50 mM Tris, 40 mM glycine, 20% methanol, Bio-Rad Trans-Blot Turbo Semi-Dry Cell, 25 V, 30 min) onto a transfer membrane (Immobilon-P^{SQ} 0.2 µm pore size). The membrane was blocked in 5% nonfat milk in TBST (0.1% Tween-20 in Tris buffered saline) for 1 hour at room temperature. The membrane was incubated in anti-Arf6 (1:300) in 5% milk in TBST overnight at 4 °C with gentle agitation followed by washing with TBST (3 x 10 min). Blots were then treated with anti-rabbit-HRP conjugated secondary antibody (1:10000) in TBST for 1 hour, washed with TBST (3 x 10 min), and visualized with Clarity Western ECL Substrate (Bio-Rad). After imaging, the antibodies were stripped by submerging the membrane in Restore Western Blot Stripping Buffer for 30 min. The membrane was then washed 3 times in TBST, loaded into a Snap i.d. 2.0 cassette (Millipore Sigma), rapidly blocked with 0.5 % milk in PBST, blotted with anti-HA-HRP conjugated antibody (1:1000) for 10 min and washed 4 x with PBST using the Snap i.d platform. The blot was again visualized for HA-NOD2. The area of Co-immunoprecipitated Arf6 bands were measured using Image Lab software (Bio-rad). Boxes were drawn around Arf6 bands and around 3 random empty background areas on the blot image. Background was subtracted from the Arf6 band intensity and values were normalized to conditions with NOD2-WT, Arf6-WT and MDP. Values were averaged in triplicate from independent transfections. P-values were calculated with unpaired students t-test using Prism 9 software.

NOD2 dependent NF-□B luciferase reporter assays. 0.15 x 10^6 HEK 293T cells per well were seeded overnight in 12-well plates with complete DMEM. The following day, cells were transfected with plasmids

expressing firefly luciferase (PRDII-4x-luc 50 ng/well), Renilla luciferase (pRL-TK 50 ng/well), HA-NOD2 (50 ng/well), and 2.5 μL/well Lipofectamine 2000. After 6 hours, media was aspirated out and replaced with Opti-MEM containing MDP or L18-MDP. Cells were incubated for 2 hours. Cells were harvested, lysed and assayed for luciferase activity using Dual-Luciferase reporter kit.

II. Supplementary Figures

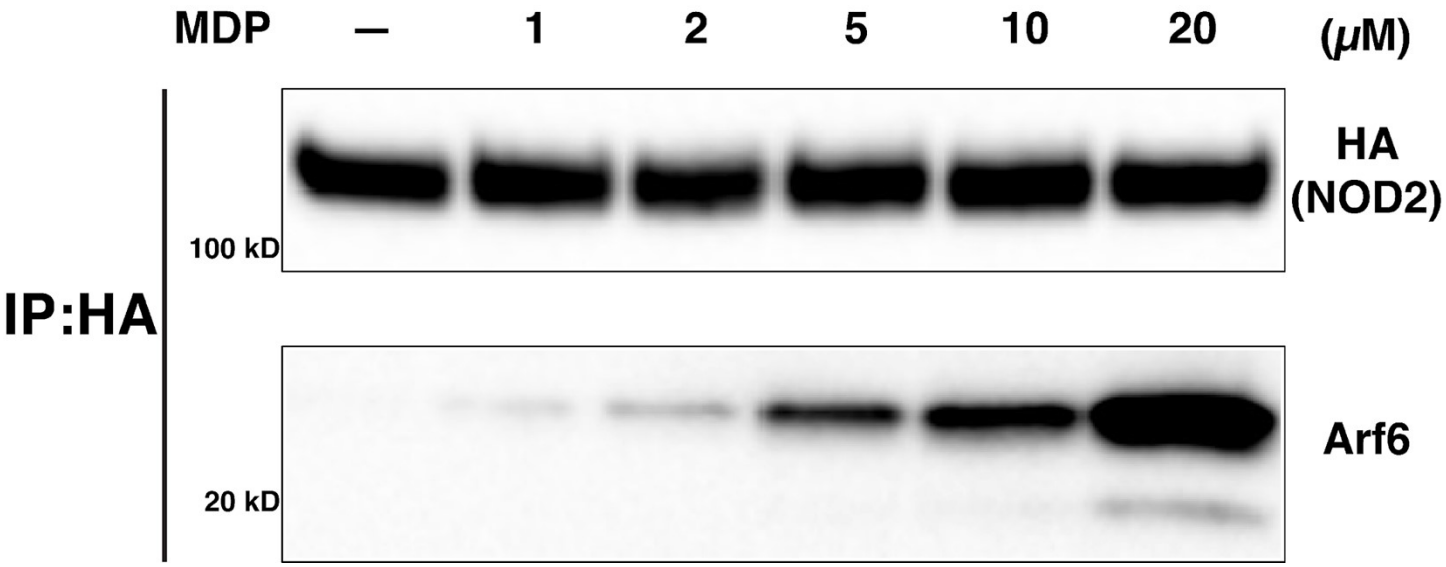

Figure S1. IP:HA of HA-NOD2 co-transfected with Arf6-myc. Arf6 co-IP increases with concentration of MDP.

III. References

1 B. Kim, Y.-C. Wang, C. W. Hespen, J. Espinosa, J. Salje, K. J. Rangan, D. A. Oren, J. Y. Kang, V. A. Pedicord and H. C. Hang, *Cell Microbiol*, , DOI:10.7554/elife.45343.
2 Y.-C. Wang, N. P. Westcott, M. E. Griffin and H. C. Hang, *ACS Chemical Biology*, 2019, 14, acschembio.8b01038-414.