Supplementary Information

BANK1 interacts with TRAF6 and MyD88 in innate immune signaling in B cells

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Supplementary Information

Supplementary Figure Legends

Supplementary Figure S1. BANK1-FL and -D2 contain TRAF6 consensus binding motif P-X-E-X-X-X-aromatic (Ar) / acidic (Ac) residue. A. Amino acid sequence of BANK1-FL protein is shown with exon 2 highlighted in blue and the W40C mutation in red. The four TRAF6 binding motifs present in BANK1 are underlined where binding motif 1 and 2 overlap. In addition, the three-core binding amino acids proline (P), glutamate (E) and glutamate or tyrosine (Y), in the consensus binding motif are shown in bold. **B.** Amino acid sequence of BANK1-D2 is shown with the four TRAF6 binding motifs already present in BANK1-FL. The motif that was formed due to the lack of exon 2 is shown in red with the core amino acids of the binding motif in bold. X means any amino acid within the TRAF6 consensus binding motif.

Supplementary Figure S2. Subcellular localization analyses of BANK1 proteins, MyD88 and TRAF6 in nonstimulated U2OS cells. A-E. U2OS cells were single transfected with BANK1-FL (**A**), BANK1-D2 (**B**), BANK1-40C (**C**), MyD88 (**D**) and TRAF6 (**E**) prior to performing double and triple co-localization analyses. DAPI was used for nuclear staining (**A**,**B**). All three BANK1 proteins (**A-C**) displayed a homogenous cytoplasmic distribution with some small punctuate accumulations. MyD88 (**D**) displayed various unique aggregate-like patterns whereas TRAF6 (**E**) showed a cytoplasmic distribution similar to BANK1 proteins. Scale bars = 20 μm.

Supplementary Figure S3. Co-localization analyses of BANK1 proteins with TRAF6 and MyD88 in nonstimulated U2OS cells. A. Double transfections of BANK1-40C-YFP and RFP-TRAF6. **B.** Quantitative analyses of colocalization events of TRAF6 with BANK1-FL (n = 67) and BANK1-D2 (n = 78) using Pearson's coefficient. The Mann-Whitney unpaired t-test was applied for statistical analyses of data from BANK1 – TRAF6 colocalization with $p = .003$ (**) **C.** RFP-TRAF6 and MyD88-CFP vectors were transfected into nonstimulated U2OS cells. Note that MyD88-CFP is shown in green for a better appreciation of merged images. **D.** Triple transfections of BANK1-40C-YFP with MyD88-CFP and RFP-TRAF6. Scale bars = 15 μm (**C**) and 10 μm (**B**). Note that BANK1-40C-YFP is shown in green for a better appreciation of merged images.

Supplementary Figure S4. Graphic presentation of fold changes for BANK1 and MyD88 interaction with TRAF6 in Namalwa B cells. A. and B. Endogenous CoIP analyses in the Namalwa B cell line were performed by immunoprecipitating TRAF6 and interrogating for interaction with anti-BANK1 and anti-MyD88 antibodies in the Namalwa B cell line. Western blot bands of BANK1 and MyD88 were normalized by housekeeping protein β-Actin using ImageJ program. Time point 0 was set as 1.0. Subsequent fold changes in BANK1 and MyD88 interaction with TRAF6 are shown as scatter dot blot in the graph derived from cells induced with **A.** TLR7 agonist R848 and **B.** TLR9 agonist ODN 2006 from at least three separate experiments. Means for each data set are shown as horizontal lines. **C.** Table of row statistic analyses for BANK1 and MyD88 from R848 and below from ODN 2006 stimulated Namalwa B cells as means+SEM. n, number of experiments.

Supplementary Figure S5. Confirmation of pathway activation. A. IL-8 and TNF-α production was measured by ELISA in the Namalwa B cell line after 24 h stimulation with TLR7 agonist R848 and TLR9 agonist ODN 2006. **B.** IL-6 production was measured in purified splenic mouse B cells by ELISA 24 h and 48 h post-stimulation with TLR7 agonist R848 and TLR9 agonist ODN 1826. The Mann-Whitney test was applied for statistical analyses of data with p .12 (ns), p .033 (*), p .002 (**), $p < .001$ (***). ns, not significant.

Supplementary Figure S6. Single immunofluorescence in nonstimulated (NS) mouse B cells and Amnis analyses in nonstimulated, TLR7 (agonist R848) and TLR9 (agonist ODN 1826) 30 min stimulated mouse total CD19$^+$ cells. A. Image flow cytometry on purified mouse CD19$^+$ cells from single immunofluorescence reactions against MyD88 (goat anti-rabbit 488, boxed in green), BANK1 (goat anti-mouse Alexa Fluor 647, boxed in red), TRAF6 (goat anti-mouse 488, boxed in green), and TLR9 (donkey anti-goat Alexa Fluor 555, boxed in orange) to exclude leakage of signals to adjacent channels. Nuclei were stained with Hoechst 33342 as shown in Channel 6. **B.** The table shows an overview of the bright detail similarity feature R^3 for cells stained in immunofluorescence reactions against the proteins listed. The R^3 are means from three independent experiments with each SEM. The Wizard for colocalization in the Amnis program was applied to measure the degree of colocalization for cells double positive for two probes under examination. We determined colocalization for all conditions which is defined as $R^3 > 1$. Scale bars = 7 µm.

Supplementary Figure S7. Confocal analyses in nonstimulated mouse B cells and Amnis analyses in nonstimulated, TLR7 (agonist R848) and TLR9 (agonist ODN 1826) 30 min stimulated mouse total CD19^{+} cells. A. Confocal microscopy image from double immunofluorescence reaction against mouse BANK1 (goat anti-mouse Alexa Fluor 647) with rabbit MyD88 (goat anti-rabbit Alexa Fluor 488) and rabbit BANK1 (goat anti-rabbit Alexa Fluor 647) with mouse TRAF6 (goat anti-mouse Alexa Fluor 488) on mouse purified B cells. **B and C** Analyses of % of double positive total mouse CD19^{+} cells for MyD88 – TLR7 and TRAF6 – TLR7 (**B**) and MyD88 – TLR9 and TRAF6 – TLR9 (**C**). One-way ANOVA test was applied to determine significant changes with p .12 (ns), p .33 (*), p .002 (**). ns, not significant. Scale bars = 10 μm.

Supplementary Figure S8. Graphic presentation of fold changes for BANK1 mutant interaction with TRAF6. A and B. Shown are fold changes of the interaction of BANK1-FL mutants (**A**) and BANK1-D2 (**B**) with TRAF6 relative to control reaction BANK1-FL/D2-WT-V5 with Myc-TRAF6. The ratio of this reaction was set as 1.0. Fold changes and their means+SEM are shown in a scatter dot plot with at least 7 dots representing each an independent experiment. The Wilcoxon signed-rank test was used for statistical analyses of BANK1 mutants with p .12 (ns), p .033 (*), p .002 (**), $p < .001$ (***). ns, not significant.

Supplementary Figure S9. Graphic presentation of peptide experiments. A and B. Shown are ratios of **A.** BANK1-FL-V5 and **B.** BANK1-D2-V5 and Myc-TRAF6 obtained from Western blot analyses which tested BANK1 peptides for their ability and specificity to interfere with BANK1-TRAF6 interaction. Concentration of peptides tested are shown on X-axis. Ratios are shown in a scatter dot plot with at least 3 dots representing each an independent experiment and their means+SEM. The statistical significance for each concentration and peptide are shown. A nonparametric t-test (Mann-Whitney) was applied to determine significant changes with *p* 0.1234 (ns), 0.0332 (*), 0.0021 (**), 0.0002 (***), < 0.0001 (****). ns, not significant.

Supplementary Figure S10. Polyubiquitination of BANK1 in nonstimulated HepG2 cells. BANK1 proteins were tested for their general ubiquitination status (HA-Ub) that expresses all 7 types of ubiquitin proteins and for K48-linked polyubiquitination in HepG2 cells with 1 µg HA-Ub or HA-K48 and 4 µg BANK1-V5 vectors and performing the pulldowns with an anti-HA antibody. First four lanes are control CoIPs to visualize background signals.

Supplementary Figure S11. Transient expression of BANK1-V5 proteins and Myc-TRAF6 in HepG2 cells. Cells were transfected with 0.1 µg pDNA pRK5-Myc-TRAF6 and various 0.4 µg pcDNA.3.1-BANK1-V5 vectors per 24-well plates. HepG2 cells were left nonstimulated or stimulated with 2 µM ODN 2006 and 8 µg/ml R837 and supernatants were collected 72 h afterwards to perform ELISA assays for IL-8. While supernatants were taken for ELISAs, nonstimulated cells were also harvested at 72 h post-stimulation, lysed, western blots were run and immunoblottings against V5 and Myc were performed to check for transient transfection efficiency. Anti β-actin antibody was used as loading control.

Supplementary Figure S1

Putative TRAF6 Binding Motifs: P-X-E-X-X-X-Ar/Ac and P-X-E-X-X-Ar/Ac

a

>NP_060405.4 B-cell scaffold protein with ankyrin repeats isoform 1 [Homo sapiens]

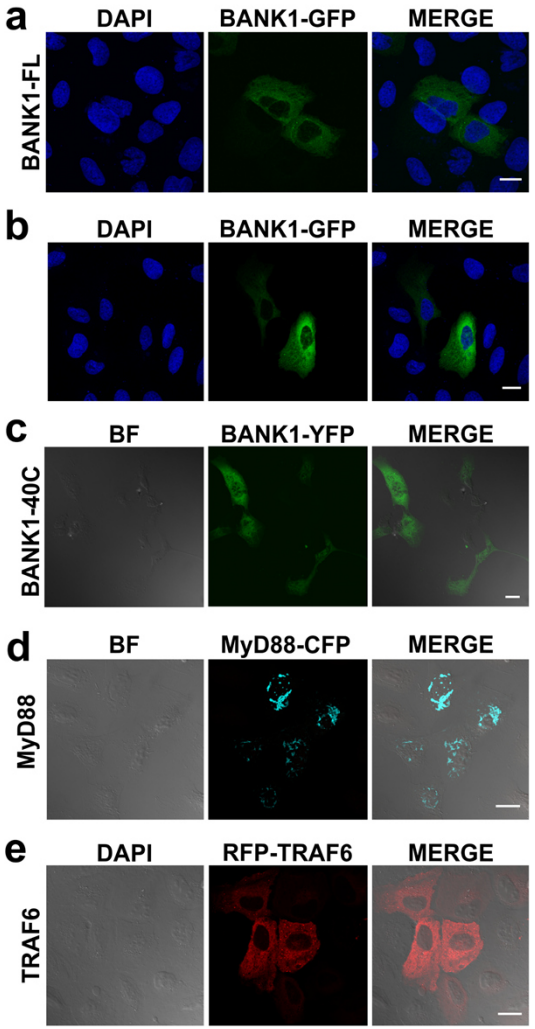
MLPAAPGKGLGSPDPAPCGPAPPGNTKDIIMIYEEDAEEWALYLTEVFLHVVKREAILLYRLENFS
FRHLELLNLTSYKCKLLILSNSLLRDLTPKKCQFLEKILHSPKSVVTLLCGVKSSDQLYELLNISQ
SRWEISTEQEPEDYISVIQSIIFKDSEDYFEVNIPTDLRAKHSGEISERKEIEELSEASRNTIPLA
VVL**PTEIPCE**NPG**E**IFIILRDEVIGDTVEVEFTSSNKRIRTRPALWNKKVWCMKALEFPAGSVHVN
VYCDGIVKATTKIKYYPTAKAKECLFRMADSGESLCQNSIEELDGVLTSIFKHEIPYYEFQSLQTE
ICSQNKYTHFKELPTLLHCAAKFGLKNLAIHLLQCSGATWASKMKNMEGSDPAHIAERHGHKELKK
IFEDFSIQEIDINNEQENDYEEDIASFSTYIPSTQNPAFHHESRKTYGQSADGAEANEMEGEGKQN
GSGMETKHS**PLE**VGS**E**SSEDQYDDLYVFIPGADPENNSQEPLMSSRPPLPPPRPVANAFQLERPHF
TLPGTMVEGQMERSQNWGHPGVRQETGDEPKGEKEKKEEEKEQEEEEDPYTFAEIDDSEYDMILAN
LSIKKKTGSRSFIINRPPAPTPRPTSIPPKEETTPYIAQVFQQKTARRQSDDDKFRGLPKKQDRAR
IESPAFSTLRGCLTDGQEELILLQEKVKNGKMSMDEALEKFKHWQMGKSGLEMIQQEKLRQLRDCI
IGKR**PEE**ENV**Y**NKLTIVHHPGGKETAHNENKFYNVHFSNKLPARPQVEKEFGFCCKKDH

b

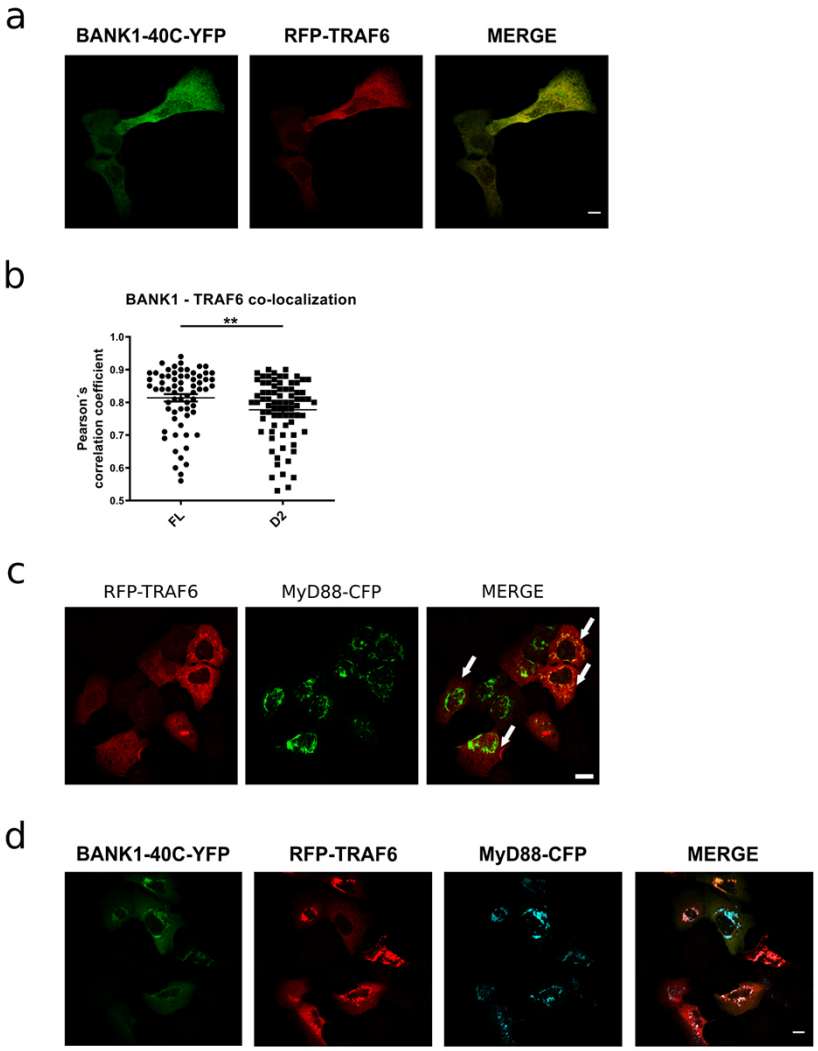
>NP_001120979.2 B-cell scaffold protein with ankyrin repeats isoform 3 [Homo sapiens]

MLPAAPGKGLGSPDPAPCGPA**PPDSEDY**FEVNIPTDLRAKHSGEISERKEIEELSEASRNTIPLAV
VL**PTEIPCE**NPG**E**IFIILRDEVIGDTVEVEFTSSNKRIRTRPALWNKKVWCMKALEFPAGSVHVNV
YCDGIVKATTKIKYYPTAKAKECLFRMADSGESLCQNSIEELDGVLTSIFKHEIPYYEFQSLQTEI
CSQNKYTHFKELPTLLHCAAKFGLKNLAIHLLQCSGATWASKMKNMEGSDPAHIAERHGHKELKKI
FEDFSIQEIDINNEQENDYEEDIASFSTYIPSTQNPAFHHESRKTYGQSADGAEANEMEGEGKQNG
SGMETKHS**PLE**VGS**E**SSEDQYDDLYVFIPGADPENNSQEPLMSSRPPLPPPRPVANAFQLERPHFT
LPGTMVEGQMERSQNWGHPGVRQETGDEPKGEKEKKEEEKEQEEEEDPYTFAEIDDSEYDMILANL
SIKKKTGSRSFIINRPPAPTPRPTSIPPKEETTPYIAQVFQQKTARRQSDDDKFRGLPKKQDRARI
ESPAFSTLRGCLTDGQEELILLQEKVKNGKMSMDEALEKFKHWQMGKSGLEMIQQEKLRQLRDCII
GKR**PEE**ENV**Y**NKLTIVHHPGGKETAHNENKFYNVHFSNKLPARPQVEKEFGFCCKKDH

Supplementary Figure S2

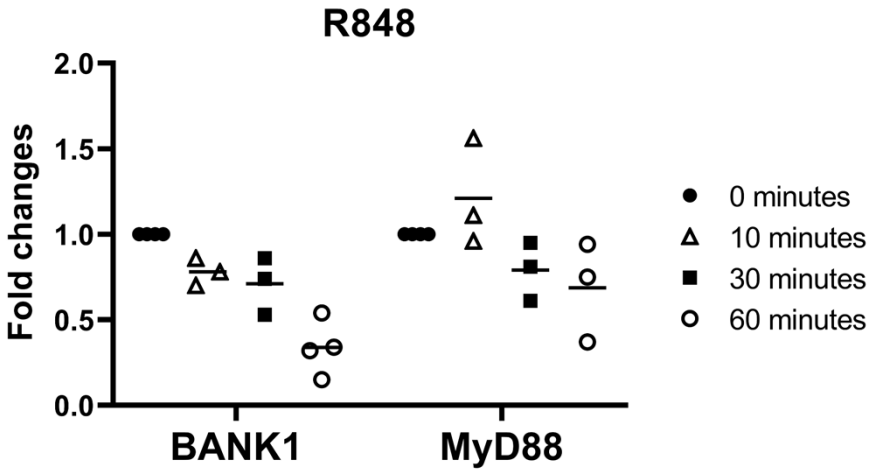


Supplementary Figure S3

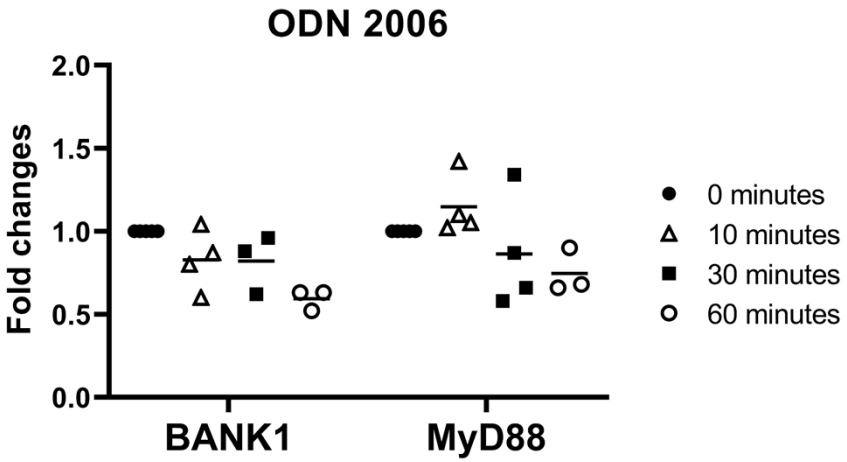


Supplementary Figure S4

a



b



c

Row statistics of CoIP bands from Western blot analyses of BANK1 and MyD88

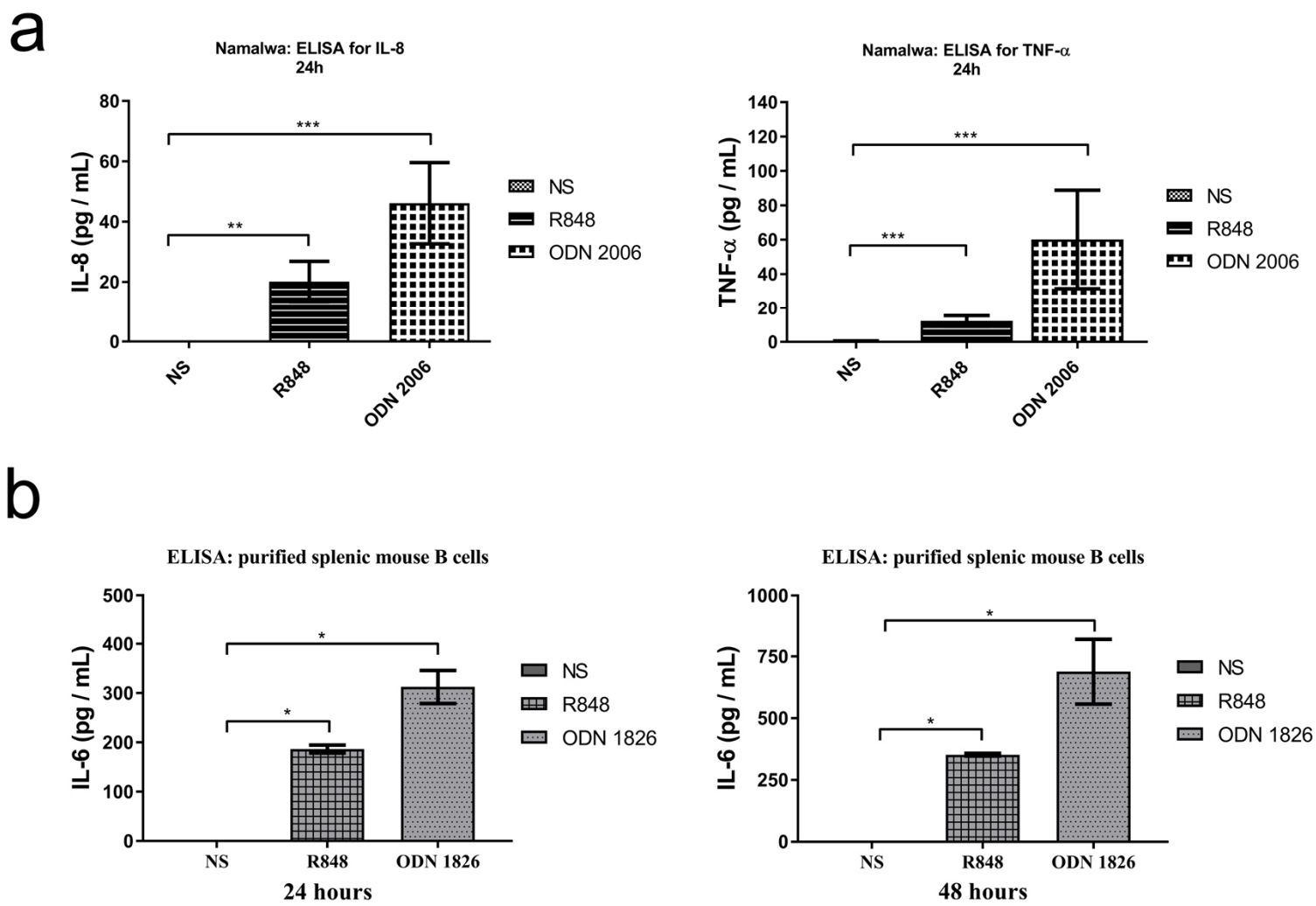
Resiquimod R848

	0 minutes			15 minutes			30 minutes			60 minutes		
	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
BANK1	1,00	0,00	4	0,780	0,046	3	0,71	0,10	3	0,34	0,08	4
MyD88	1,00	0,00	4	1,210	0,180	3	0,79	0,10	3	0,69	0,17	3

CpG B ODN 2006

	0 minutes			15 minutes			30 minutes			60 minutes		
	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N	MEAN	SEM	N
BANK1	1,00	0,00	5	0,83	0,09	4	0,82	0,10	3	0,59	0,04	3
MyD88	1,00	0,00	5	1,15	0,09	4	0,86	0,17	4	0,75	0,08	3

Supplementary Figure S5



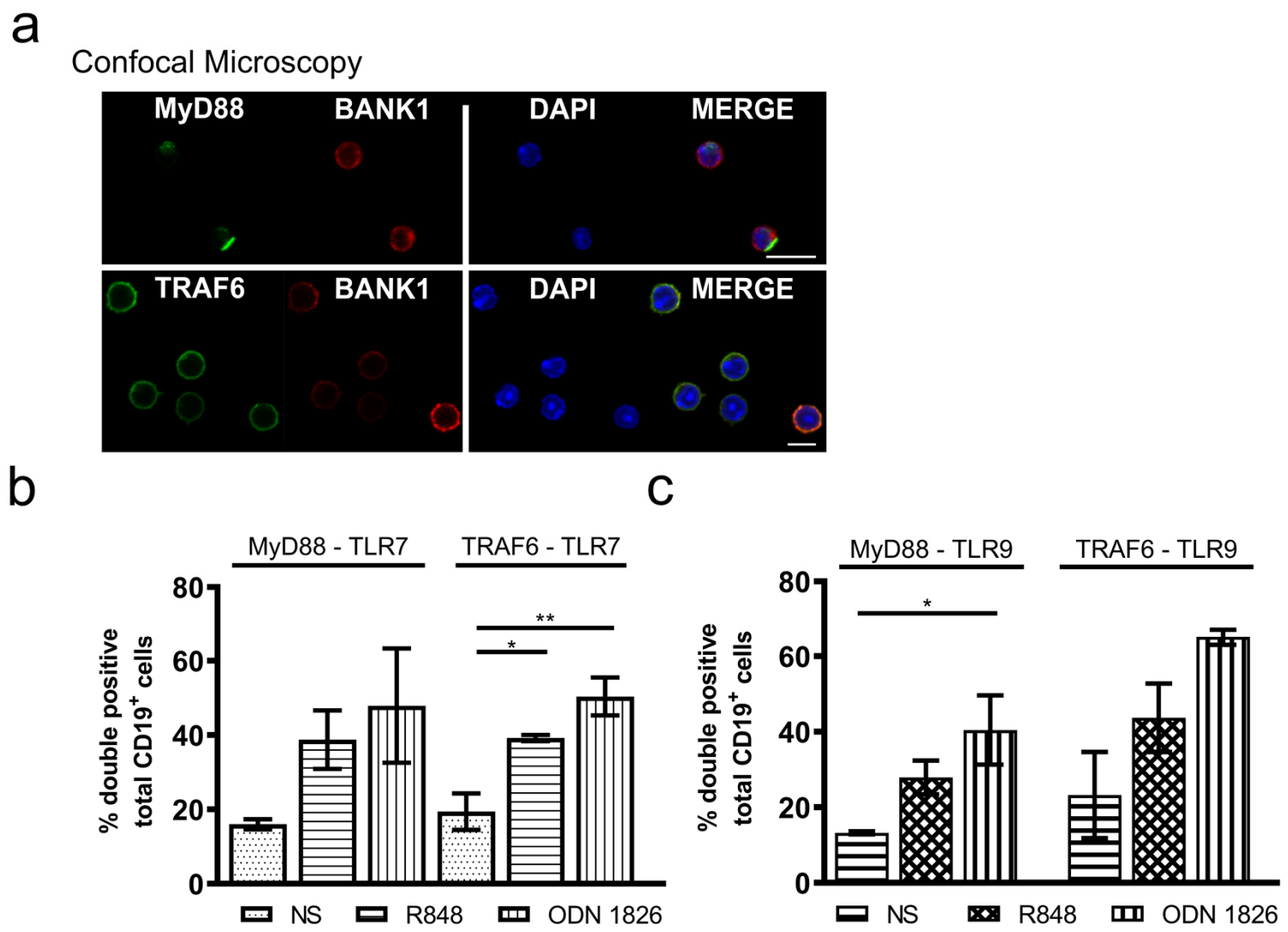
Supplementary Figure S6

a Amnis single immunofluorescences

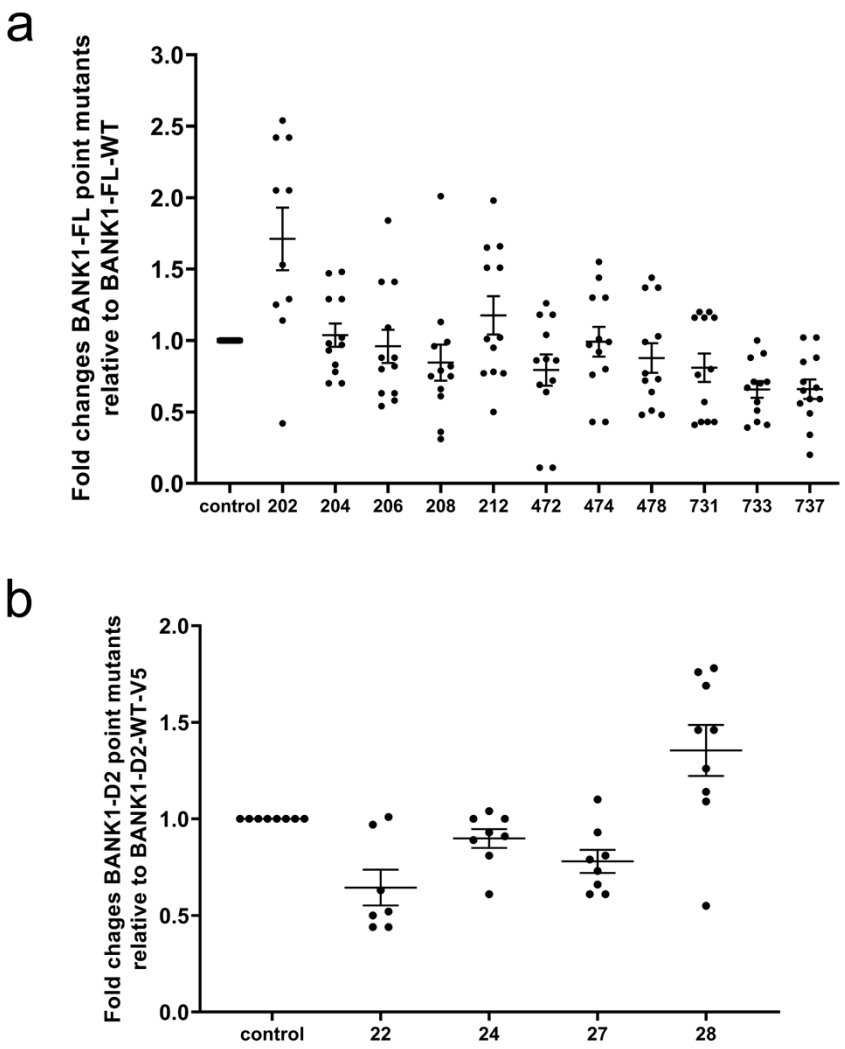
b

Proteins	R^3: NS (SEM)	R^3: R848 (SEM)	R^3: ODN 1826 (SEM)
BANK1 +TRAF6	1.59 (0.044)	1.48 (0.023)	1.47 (0.084)
BANK1 + MyD88	1.76 (0.143)	1.66 (0.123)	1.77 (0.154)
BANK1 + TLR7	2.02 (0.306)	1.88 (0.295)	1.92 (0.315)
BANK1 + TLR9	1.99 (0.311)	1.84 (0.268)	1.91 (0.293)
TRAF6 + TLR7	2.38 (0.591)	2.09 (0.437)	2.18 (0.482)
MyD88 + TLR7	2.42 (0.499)	2.23 (0.454)	2.41 (0.491)
TRAF6 + TLR9	2.29 (0.534)	2.17 (0.523)	2.12 (0.440)
MyD88 + TLR9	2.18 (0.416)	1.94 (0.329)	2.20 (0.388)

Supplementary Figure S7



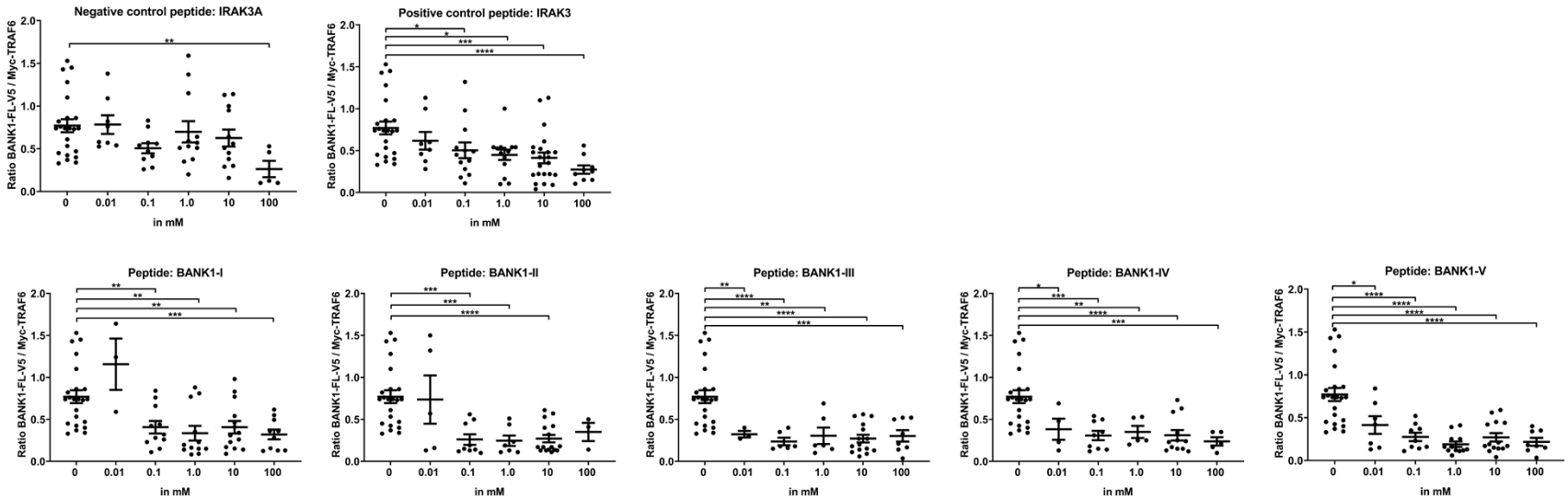
Supplementary Figure S8



Supplementary Figure S9

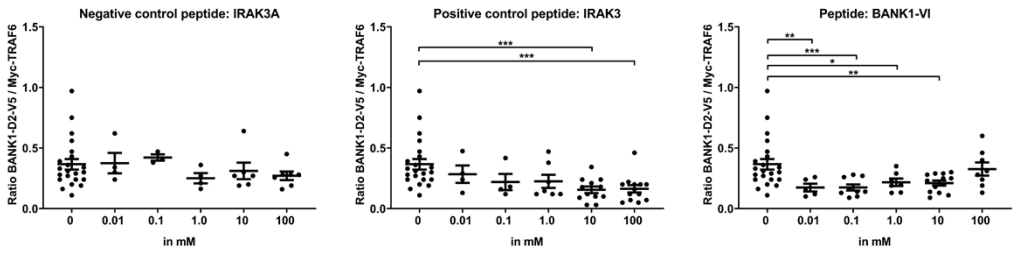
a

BANK1-FL

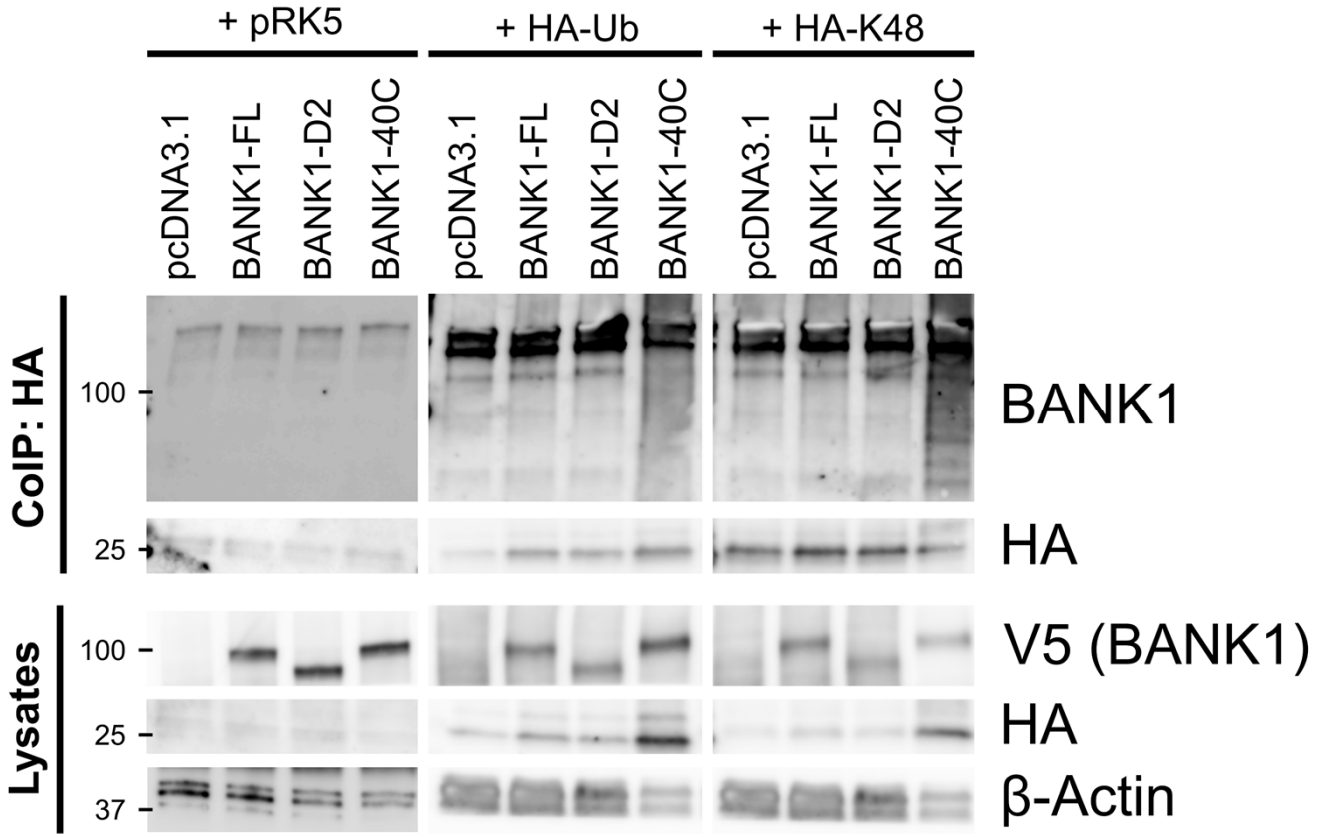


b

BANK1-D2



Supplementary Figure S10



Supplementary Figure S11