Supplemental Inventory

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Supplemental Figures

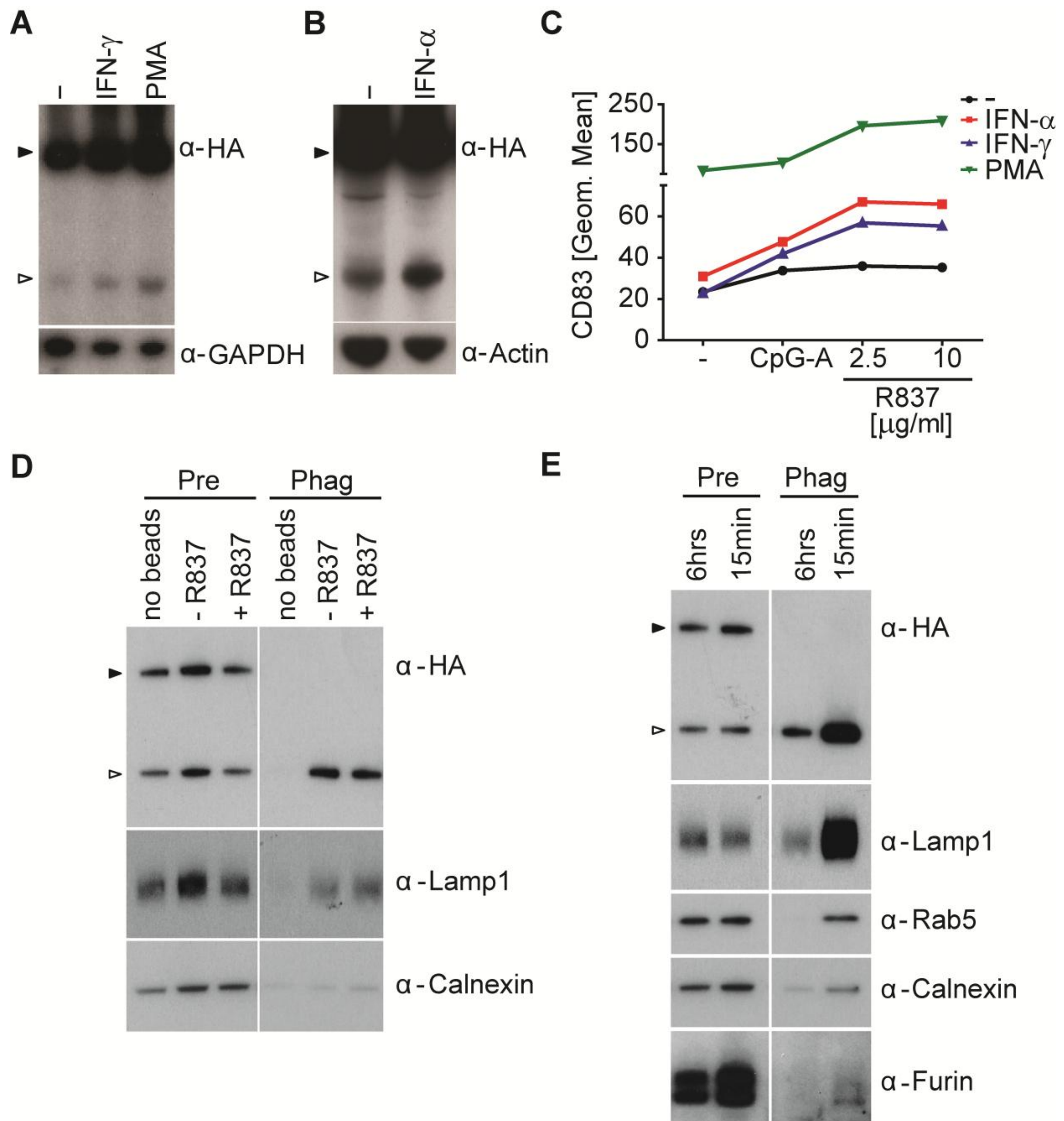


Figure S1

Pro-inflammatory Stimuli Induce Responses to TLR7 Agonists in B cells, Related to Figure 2

(A, B) Western blots corresponding to quantification results depicted in Figure 2A and 2B. Total cell lysates obtained from THP-TLR7 treated for 24 hrs with 10 nM PMA or 200 U/ml IFN-γ (A), or with 1000 U/ml IFN-α (B) were analyzed by Immunoblotting with anti-HA. Representative of at least three independent experiments. (C) $CD19^{+}$ B cells isolated by magnetic selection were treated for 24 hrs with 10 nM PMA, 200 U/ml IFN-γ, or 1000 U/ml IFN-α. After washing cells were stimulated for 24 hrs with anti-human Fab ± CpG-A (5 µg/ml) or R837 at indicated concentrations and upregulation of cell surface expressed CD83 was analyzed by flow cytometry. Representative of two independent experiments. (D) Western blot of lysates of pre-gradient controls (Pre) or phagosome preparations (Pha) from PMA differentiated THP-TLR7 cells that were fed latex beads for 6 hrs in the presence or absence of R837. Blots were probed with antibodies specific for the indicated proteins. (E) Western blot of lysates of pre-gradient controls (Pre) or phagosome preparations (Pha) from PMA differentiated THP-TLR7 cells fed latex beads in the presence R837. Cells were incubated with latex beads for 15 min and processed immediately or after a subsequent incubation (chase) for 6 hrs in total. Blots were probed with antibodies specific for the indicated proteins. Representative of three independent experiments. Full-length and truncated TLR7 are shown by filled and open arrowheads respectively.

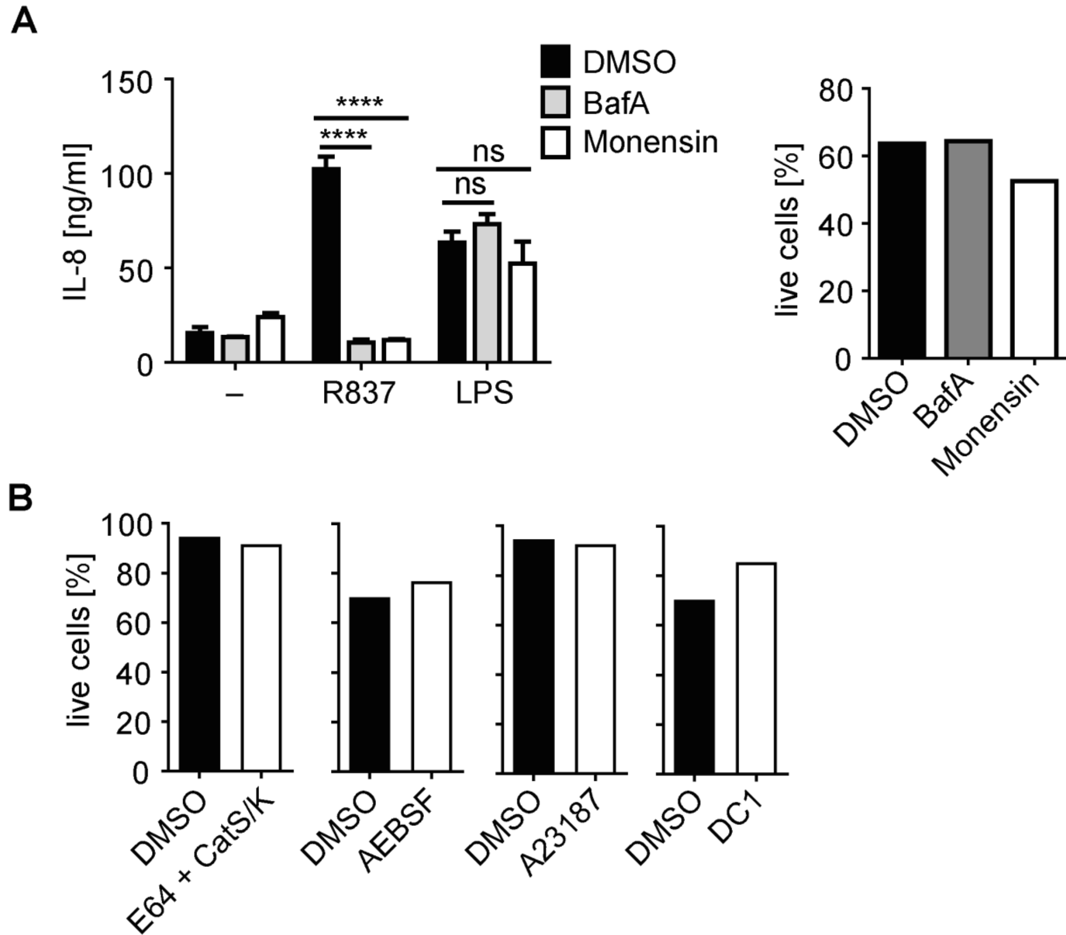


Figure S2

Low pH is Required for Responses to TLR7 Agonists, Related to Figure 3

(A) THP-TLR7 were differentiated with PMA for 24 hrs, treated with Monensin or Bafilomycin A for 4 hrs, and then stimulated with R837 or LPS for another 12 hrs. IL-8 content of lysed cells was measured using an ELISA (left panel). Values are mean ± S.D. of triplicates. ns, non significant; ****, $p < 0.0001$ by Student's t test versus control. Flow cytometry analysis of propidium iodide exclusion was performed to evaluate percentage of live cells (right panel). (B) Flow cytometry analysis of propidium iodide exclusion was performed to evaluate percentage of live cells after treatment of THP-TLR7 with the indicated inhibitors for 24 or 48 hrs as described in the relevant sections. Representative of at least three independent experiments.

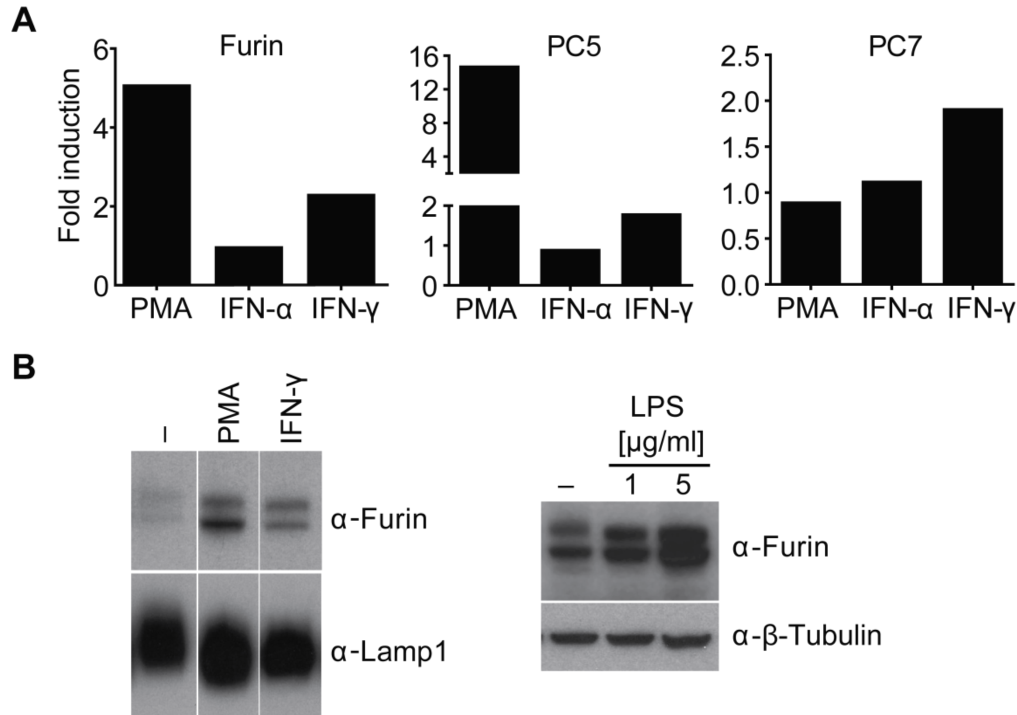


Figure S3

Pro-inflammatory Stimuli Induce Upregulation of Furin-like PCs in THP-1 cells, Related to Figure 4

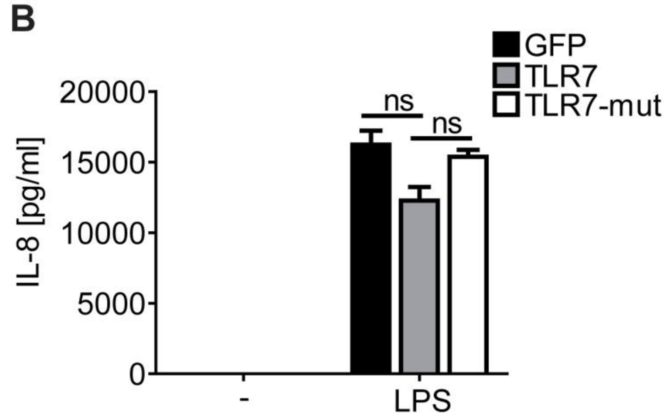
(A) THP-1 cells were treated for 24 hrs with 10 nM PMA, 200 U/ml IFN-γ, or 1000 U/ml IFN-α. Expression levels of Furin, PC5 and PC7 were analyzed by Q-PCR and normalized to GAPDH. (B) Western blots of cell lysates of THP-1 cells treated for 24 hrs with 10 nM PMA, 200 U/ml IFN-γ, or LPS at indicated concentrations were probed with antibodies against furin and LAMP-1 or tubulin as loading controls. Representative of at least three independent experiments.

A

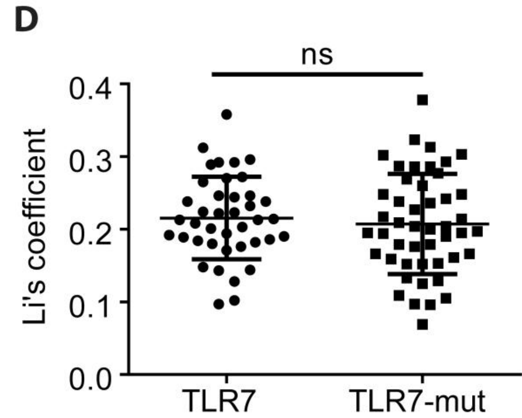
Furin-like PC recognition site:[R/K]-[X]-[R/K]↓
(X: any amino acid residue, n= 0, 2, 4, or 6)

TLR7: KQFKRLKVIDLSVNKISPSGDSSEVGFCSNARTSVESYEPQVLEQLHYFRYDKYARSCRFKNKEASFMSVN
TLR7-mut: KQFKRLKVIDLSVNKISPSGDSSEVGFCSNARTSVESYEPQVLEQLHYFAYDKYAASCRFKNKEASFMSVN

B



D



C

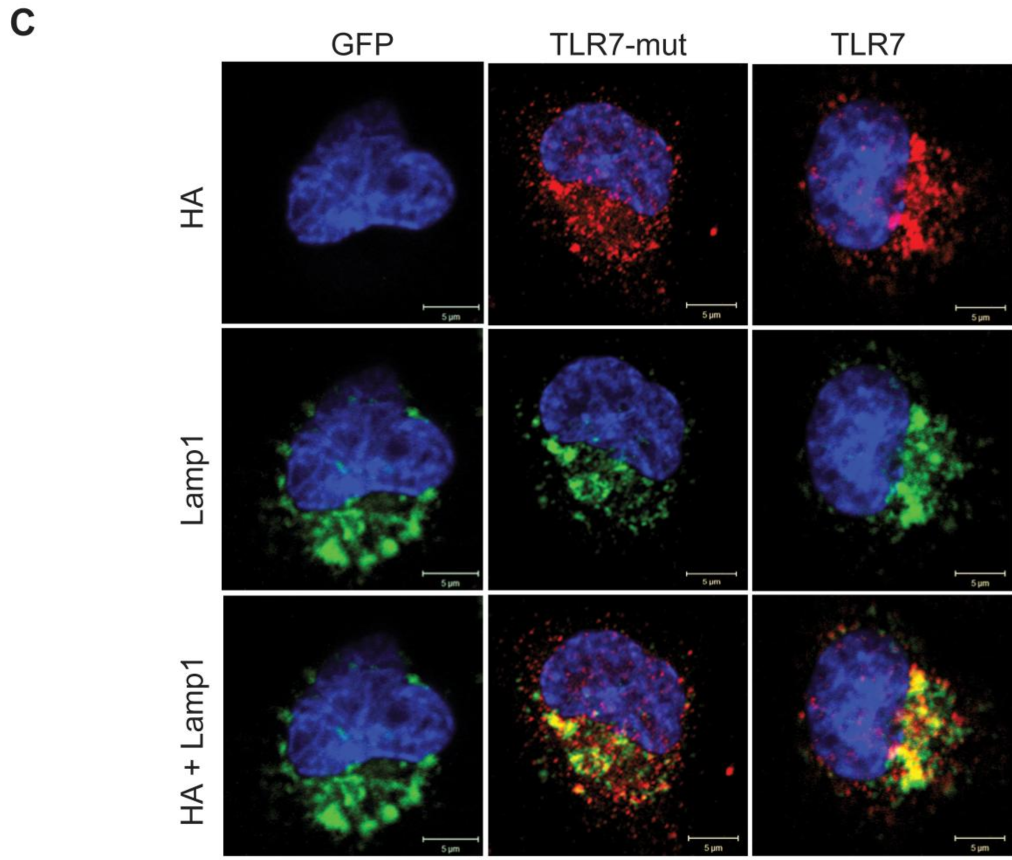
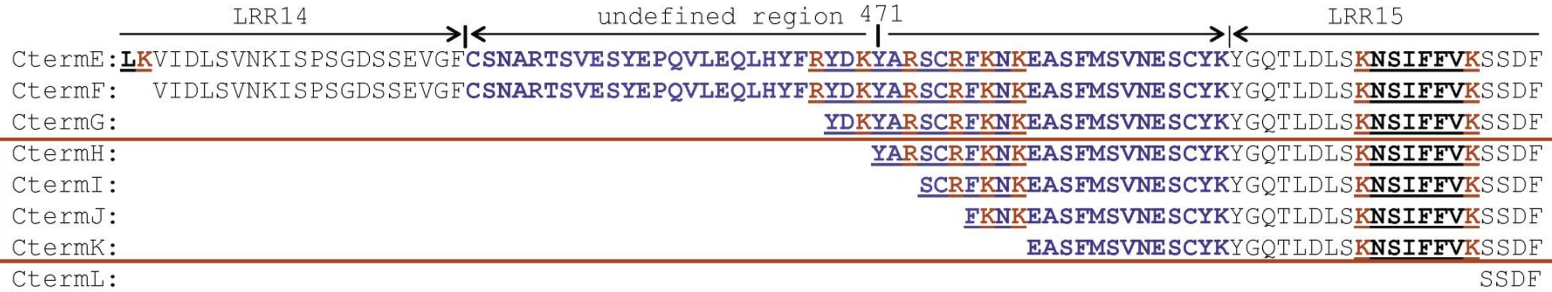
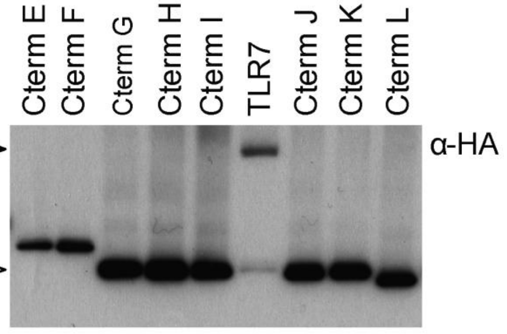


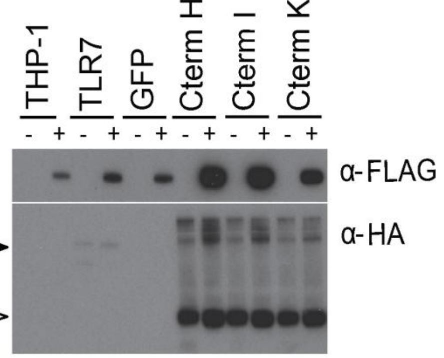
Figure S4

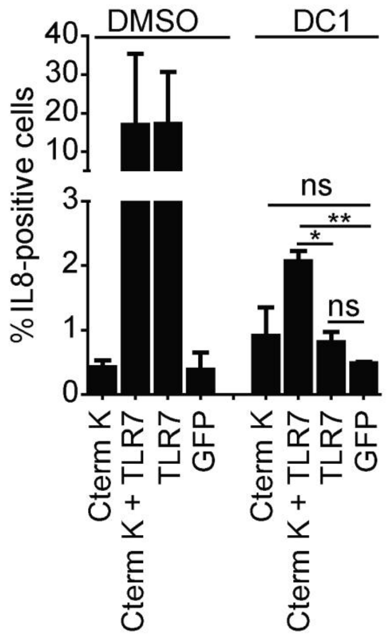
Mutant TLR7 Co-localizes with the Lysosomal Marker Lamp1, Related to Figure 5

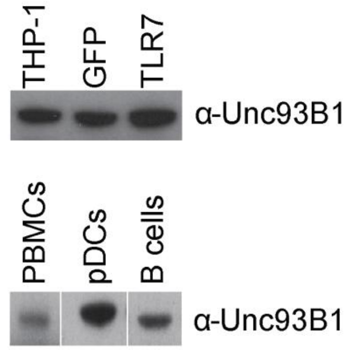
(A) Alignment of the region encompassing the potential PC recognition sites close to the undefined region of hTLR7 with the sequence of TLR7-mut. Arginine and Alanine residues exchanged by site-directed mutagenesis are shown in green. (B) Response to LPS is not compromised in THP-1 cells expressing the mutant version of TLR7. IL-8 production by the indicated cell lines was monitored by ELISA after 24 hrs of stimulation with LPS. Values are mean ± S.D. of triplicates. Representative of at least three independent experiments. (C, D) THP-1 expressing the control plasmid, the mutant version of TLR7 or the WT receptor were differentiated with PMA, fixed with PFA and stained with an anti-HA antibody and anti-Lamp1 antibody for lysosomal staining. Confocal pictures were taken using a Zeiss LSM 5. Bars represent 5 µM. (D) Li´s coefficient was calculated using Coloc2 in Fiji (Li et al., 2004; Rasband, 1997-2012). Values are mean ± S.D. ns, non significant versus control by Student's t test. Pooled data of 4 representative experiments (n ≥ 40).


A
LRR14
undefined region
471
LRR15
CtermE: LKVIDLSVNKISPSGDSSEVGFCSNARTSVESYEPQVLEQLHYFRYDKYARSCRFKNKEASFMSVNESCYKYGQTLDLSKNSIFFVKSSDF
CtermF: VIDLSVNKISPSGDSSEVGFCSNARTSVESYEPQVLEQLHYFRYDKYARSCRFKNKEASFMSVNESCYKYGQTLDLSKNSIFFVKSSDF
CtermG: YDKYARSCRFKNKEASFMSVNESCYKYGQTLDLSKNSIFFVKSSDF
CtermH: YARSCRFKNKEASFMSVNESCYKYGQTLDLSKNSIFFVKSSDF
CtermI: SCRFKNKEASFMSVNESCYKYGQTLDLSKNSIFFVKSSDF
CtermJ: FKNKEASFMSVNESCYKYGQTLDLSKNSIFFVKSSDF
CtermK: EASFMSVNESCYKYGQTLDLSKNSIFFVKSSDF
CtermL: SSDF


B
Cterm E
Cterm F
Cterm G
Cterm H
Cterm I
TLR7
Cterm J
Cterm K
Cterm L
α-HA


D
THP-1
TLR7
GFP
Cterm H
Cterm I
Cterm K
- + - + - + - + - + - +
α-FLAG
α-HA


F
DMSO
DC1
% IL8-positive cells
40
30
20
10
3
2
1
0
ns
**
*
ns
Cterm K
Cterm K + TLR7
TLR7
GFP
Cterm K
Cterm K + TLR7
TLR7
GFP


C
THP-1
GFP
TLR7
α-Unc93B1
PBMCs
pDCs
B cells
α-Unc93B1

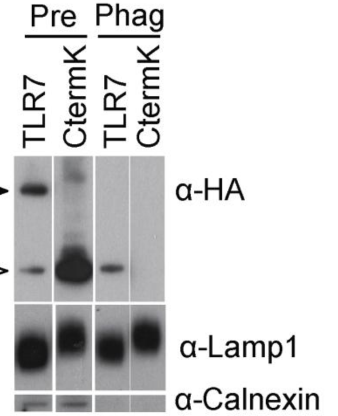

E
Pre
Phag
TLR7
CtermK
TLR7
CtermK
α-HA
α-Lamp1
α-Calnexin

Figure S5

C-Terminal Fragment of TLR7 Does Not Localize to Phagosomes, Related to Figure 6

(A) Alignment of the region encompassing the undefined region of hTLR7 with the sequences of the different truncated TLR7 molecules generated. The undefined region as predicted by Bell et al is shown in blue (Bell et al., 2003). Potential furin-like PC recognition sites are underlined. Arginine and Lysine residues within those sites are depicted in red. Red box highlights those truncated receptors that have the same molecular weight as cleaved TLR7 found in cells expressing the wild type receptor. (B) Western blot of PNGAse F treated lysates of THP-1 cells expressing the indicated constructs stained for HA-tag. (C) THP-1 cells and primary immune cells express endogenous levels of Unc93B1. Western blot of lysates of THP-1, THP-GFP and THP-TLR7 (top panel) and PBMCs, pDCs or B cells (bottom panel) probed for endogenous Unc93B1. (D) Western blot of lysates of THP-1 cells expressing the indicated constructs +/- Unc93B1-FLAG and probed for HA or FLAG. (E) Western blot of lysates of pre-gradient controls (Pre) or phagosome preparations (Phag) from PMA differentiated THP-TLR7 or -CtermK cells fed latex beads in the presence of R837. Cells were incubated with latex beads for 6 hrs in total. Blots were probed with antibodies specific for the indicated proteins. (F) Co-expression of biologically inactive full-length TLR7 restores functional activity of the TLR7 C-terminal fragment in DC1 treated cells. THP-1 cells expressing the indicated constructs were treated with DMSO or DC1 for 24 hrs and then stimulated with 10 µg/ml R837. Intracellular staining was performed to measure the percentage of IL-8 positive cells. Values are mean ± S.D. of duplicates. Full-length and truncated TLR7 are indicated by the filled and open arrowheads respectively. Representative of at least three (B - E) or two (F) independent experiments.

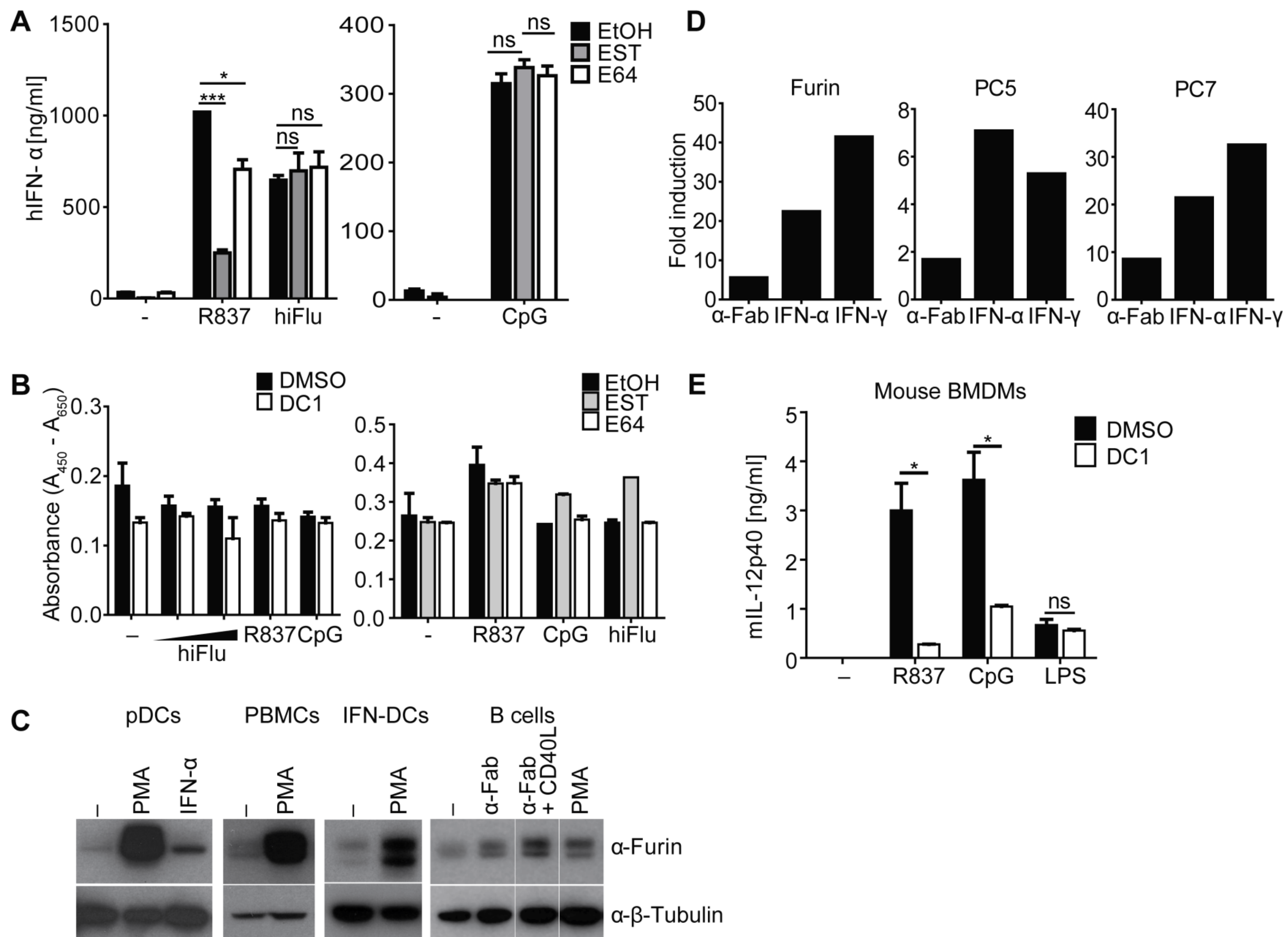

A
hIFN- α [ng/ml]
R837
hiFlu
CpG
EtOH
EST
E64
ns
B
Absorbance (A_{450} - A_{650})
DMSO
DC1
C
pDCs
PBMCs
IFN-DCs
B cells
PMA
IFN-α
α-Fab
α-Fab + CD40L
α-Furin
α-β-Tubulin
D
Furin
PC5
PC7
Fold induction
IFN-γ
E
Mouse BMDMs
mIL-12p40 [ng/ml]
LPS

Figure S6:

Pro-inflammatory Stimuli Induce Upregulation of PCs in Primary Immune cells, Related to Figure 7

(A) Enriched cultures of human pDCs were treated with EtOH, a mixture of E64 (20 μM) + Inhibitors for CatS (2 nM) and CatK (1.5 μM), or with EST (20 μM) for 24 hrs, and then stimulated with R837 (1 μg/ml), hiFlu (equivalent to MOI of 5), or CpG-A (5 μg/ml) overnight. IFN-α secretion was measured by ELISA. Histograms show mean ± SD (n = 2). Representative of at least two independent experiments. (B) WST-1 staining of pDC enriched fractions treated as indicated to evaluate toxicity of compounds and stimulation on pDC enriched fractions. Cells were analyzed after harvesting of supernatant used for ELISA shown in Fig. 7A and D. Histograms show mean ± SD (n = 2). (C) Western blots of cell lysates of indicated cells treated for 24 hrs with 10 nM PMA, or 1000 U/ml IFN-α, or with anti-human Fab alone or anti-Fab ± CD40L were stained for Furin and loading control. (D) qPCR analysis of B cells treated for 24 hrs with 10 nM PMA, 200 U/ml IFN-γ, or 1000 U/ml IFN-α. Expression levels of Furin, PC5 and PC7 were analyzed and normalized to GAPDH. Values are mean of triplicates. (E) DC1 inhibits R837 and CpG induced IL-12p40 secretion in mouse BMDMs. For ELISA analysis cells were treated with DMSO or DC1 (70 μM) for 24 hrs, and then stimulated with R837 (1 μg/ml), CpG (0.5 μg/ml) or LPS (1 μg/ml) for 24 hrs in the presence of inhibitors. mIL-12p40 secretion was measured. Values are mean ± S.D. of triplicates. ns = non significant; *, $p < 0.05$; ***, $p < 0.001$ by Student's t test versus control. (D - E) Representative of two independent experiments.

Supplemental Experimental Procedures

Reagents

Imiquimod (R837), R848, CpG-A (ODN2216), and CpG-B (ODN2006) were from Invivogen. The purified Influenza virus PR8 was kindly donated by Alain Townsend, MRC Weatherall Institute of Molecular Medicine, University of Oxford, UK. Bafilomycin A, anti-β-Tubulin (TUB 2.1), anti-Actin, Concanamycin A, DC1 (3,3'-(5-Indaylmethylene)bis(4-hydroxycoumarin)), DC2 (3,3',3'',3'''-(1,4-PHENYLENEDIMETHYLIDYNE)TETRAKIS(4-HYDROXYCOUMARIN)), EST, E-64 (trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane), LPS (Escherichia coli type), A23187 (Calcium Ionophore), and AEBSF (4-(2-aminoethyl)benzenesulfonyl fluride hydrochloride) were from Sigma-Aldrich. Cathepsin S Inhibitor and cathepsin K Inhibitor II were from Calbiochem, Monensin was from eBioscience, and PNGAse F was from New England Biolabs. Monoclonal anti-HA 3F10 and complete protease inhibitor tablets were from Roche. Anti-human CD107A (Lamp1), and anti-GAPDH were from AbD Serotec, and BioLegend, respectively. Anti-human Cathepsin B, anti-Calnexin C-20 and anti-Furin were from Santa-Cruz. CD11c-PE was from Immunotech, BDCA-2-APC, BDCA-4 and anti-APC coated magnetic beads were from Milteny Biotech. Anti-rat-HRP, anti-mouse-HRP and anti-goat-HRP were from R&D, Streptavidin-PeCy7, CD19-FITC, CD19-APC, and CD123-biotin were from BD Pharmingen™. Anti-rabbit-Alexa 555 and anti-mouse-Alexa 647 were from Invitrogen. Anti-Fab was from Jackson ImmunoResearch. For hIL-8 ELISA purified anti-hIL-8, monoclonal (Clone G265-5, #554716) and biotinylated anti-hIL-8, monoclonal (#554718), and for mIL-12p40 ELISA, purified anti-mIL-12p40 (#551219) and biotinylated anti-mIL-12p40 (#554476) from BD Pharmingen were used. To detect IFN-α, the pan-specific human IFN-α kit (MABTECH AB, 3425-1H-6) was used.

Cell Culture Media, Cell Lines, and PMA differentiation

R10: RPMI supplemented with 10% (v/v) heat-inactivated FCS, Penicillin/Streptomycin, L-Glutamine, 25 mM HEPES, Nonessential Amino Acids,

1 mM Sodium Pyruvate, 50 µM β-mercaptoethanol, and $NaHCO_3$ (Sigma). *D10:* DMEM high glucose supplemented with 10% (v/v) heat-inactivated FCS, Penicillin/Streptomycin, and L-Glutamine. Human THP-1 cells (myelomonocytic) were cultured in R10 and HEK293null (transformed kidney epithelium) and LoVo (colorectal adenocarcinoma) cells were cultured in D10. Cells were grown at 37 °C in humidified air with 5% CO_2. If not indicated otherwise, monocytic THP-1 cells were differentiated into a macrophage-like cell line by culturing them in R10 supplemented with 10 nM PMA for 24 to 72 hrs.

Generation of IFN-DCs, and pDC and B Cell Isolation

To generate human IFN-DCs from healthy donors buffy coats $CD14^+$ cells were MACS sorted from PBMCs using Miltenyi magnetic beads, and then resuspended in R10 supplemented with 50 ng/ml recombinant human GM-CSF (PreproTech) and 500 U/ml recombinant human IFN-α (PreproTech) at 5×10^5 cells/ml. IFN-α was replenished every 3 days. The cell suspension was equally distributed into 6-well plates (3 ml/well) and incubated for 5 to 6 days at 37 ºC, 5% CO_2. For isolation of pDCs, leucocytes were MACS separated by Miltenyi magnetic beads into BDCA-4^+ and BDCA-4^- fractions to enrich for pDCs according to the manufacturer's procedure. Extent of enrichment was analyzed by flow cytometry using BDCA-2-APC, CD11c-PE, CD19-FITC, and CD123-biotin + Streptavidin-PeCy7. To enrich for B cells, leucocytes were separated into $CD19^+$ and $CD19^-$ fractions using Miltenyi magnetic beads according to the manufacturer's instruction. B cell stimulation was carried out by receptor crosslinking with an anti-Fab ± CD40L or TLR agonists.

Phagosome Isolation

Forty million THP-1 cells stably expressing TLR7-HA were differentiated with 10 nM PMA for 24 hrs. The following day, cells were pulsed for 45 min at 37 °C with 80 µl of 2 µm latex beads (polybead polystyrene microspheres, Polysciences). Beads were washed away by rinsing twice with ice-cold PBS and, unless otherwise indicated, cells were stimulated with 1 µg/ml R837, and incubated for 5 - 6 hrs at 37 °C. After rigorous washing in ice-cold PBS, THP-1 cells were scraped into 3 ml

sucrose homogenization buffer (SHB, 250 mM sucrose and 3 mM Imidazole in water) and pelleted. Cells were resuspended in 1 ml SHB, plus protease inhibitor cocktail (Roche) and EDTA, and disrupted by 20 strokes in a dounce homogenizer. The disrupted cells were gently rocked for 10 min at 4 °C to free the intracellular contents and centrifuged at 8,000 g to remove intact cells, nuclei and debris. A total of 50 µl of the supernatant was set aside as pre-gradient control and lysed by adding 1% Triton X-100 and protease inhibitors. To separate intact phagosomes from the remaining supernatant, the supernatant was mixed with 1 ml of 60 % sucrose, and applied to an ice-cold SW40 tube (Ultra Clear centrifuge tubes, 14×89 mm, Beckman Coulter) stacked with a sucrose gradient as follows: 1 ml 60 % sucrose, 2 ml homogenate, 2 ml 32 % sucrose, 2 ml 20 % sucrose and 2 ml 10 % sucrose. The gradient was spun at 100,000×g for 65 min in a Beckman centrifuge with a SW41Ti rotor at 4 °C. Following centrifugation, phagosomes were harvested from the 20–10 % sucrose interface. Phagosomes were transferred to a clean SW40 tube, diluted with ice cold PBS to a final volume of 12 ml and spun at 40,000×g for 15 min at 4 °C in a SW41Ti rotor. The phagosome pellet was lysed in lysis buffer. For Western blot analysis, loading of phagosomes samples was normalized either by volume or by number of phagocytosed beads.

DNA Cloning, Site-directed Mutagenesis, Lentiviral Transduction, and Gene Knockdown

Human TLR7 tagged at the C-terminus with HA was generated by PCR with the primers 5′-AACTTGATCACTGAGATCACCGGTAGGAGG-3′ (forward) and 5′-TGGTGTCGACGCTAGCTTTAGGCGTAGTCT-3′ (reverse) using pUNO-hTLR7-HA (InvivoGen) as template. The construct was cloned into the enhanced GFP-expressing HIV based lentiviral vector pHR-SIN-IRES-Em and was verified by sequencing. The unmodified GFP expressing lentiviral plasmid was used as negative control. Double mutations were inserted using the quick QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer’s instructions. For mutagenesis of Arg 467 and Arg 473, sense primer 5′-ACATTATTTCGCATATGATAAGTATGCAGCGAGTTGCAGATTC-3′ and antisense

primer 5′-GAATCTGCAACTCGCTGCATACTTATCATATGCGAAATAATGT-3′ were used. To clone the C-terminal fragments, a new parental lentiviral vector was generated by first inserting a start codon, Kozak sequence and the leader sequence of Influenza virus HA protein upstream of the multiple cloning site of pHR-SIN-IRES-Em by annealing oligo 1 with oligo 2 (see sequences below) and inserting it into pHR-SIN-IRES-Em. The generated vector was used to insert the different C-terminal fragments which were PCR amplified using the fragment specific forward primers together with the reverse primer Cterm_R shown below. All constructs were verified by sequencing. MISSION shRNA lentiviral vectors targeting furin, PC5 or PC7 were purchased from Sigma-Aldrich. Specific TRC clones tested are listed below. Lentiviral vectors encoding FLAG-tagged Unc93B1 (EX-Y2155-Lv121) and FLAG-tagged MyD88 (EX-Z8113-Lv121) were purchased from GeneCopoeia. To produce lentiviral particles, HEK293T cells were co-transfected with plasmids encoding VSV-G and Gag-Pol (Zufferey et al., 1997), as well as the appropriate lentiviral vectors using Fugene-6 (Roche) per the manufacturer's instructions. 48 and 72 hrs after transfection, medium containing viral particles was collected and was added directly to cells for infection. Alternatively viral particles were concentrated by sucrose gradient density centrifugation (JA25.5 rotor, 24000 rpm, 4 ºC, 2h), and then added to cells. The day after transduction, cells were given fresh medium and cultured. After expanding, GFP positive cells were isolated by FACS sorting. Alternatively, puromycin resistant cells were selected 48 hrs after transduction by adding 5 to 10 μg/ml puromycin to selection medium.

Primers used for cloning of C-terminal fragments:

Oligos	**Sequence (5′- 3′)**
oligo 1	GATCACCACCATGAAGGCAAACCTACTGGTCCTGTTATGTGCACTTGCAGCTGCAGATGCAGGATCCATTTTAC
oligo 2	TCGAGTAAAATGGATCCTGCATCTGCAGCTGCAAGTGCACATAACAGGACCAGTAGGTTTGCCTTCATGGTGGT
Forward primers:	
CtermE_F	ACCATGATCACTGAAAGTCATAGATCTTTCAGTGAATAA
CtermF_F	ACCATGATCAGTCATAGATCTTTCAGTGAATAAAATATCACC
CtermG_F	ACCATGATCATATGATAAGTATGCAAGGAGTTGCAG

CtermH_F	ACCATGATCATATGCAAGGAGTTGCAGATTCA
CtermI_F	ACCATGATCAAGTTGCAGATTCAAAAACAAAGAG
CtermJ_F	ACCATGATCATTCAAAAACAAAGAGGCTTCTTTC
CtermK_F	ACCATGATCAGAGGCTTCTTTCATGTCTGTTAATG
CtermL_F	ACCATGATCATCCTCTGATTTTCAGCATCTTTCT
Reverse primer:	
Cterm_R	TGGTGTCGACGCTAGCTTTAGGCGTAGTCT

MISSION® shRNA Plasmid DNA used for silencing of furin-like PCs:

Plasmid No.	**TRC clone**
For silencing of PC7 (NM_004716):	
7.1	TRCN0000072397; NM_004716.2-783s1c1
7.2	TRCN0000072396; NM_004716.2-1635s1c1
7.3	TRCN0000072395; NM_004716.2-1276s1c1
7.4	TRCN0000072394; NM_004716.2-1029s1c1
7.5	TRCN0000072393; NM_004716.2-3024s1c1
For silencing of PC5 (NM_006200):	
5.1	TRCN0000294471; NM_006200.3-761s21c1
5.2	TRCN0000294470; NM_006200.3-2415s21c1
5.3	TRCN0000051178; NM_006200.2-2786s1c1
5.4	TRCN0000051179; NM_006200.2-2290s1c1
5.5	TRCN0000051182; NM_006200.2-838s1c1
For silencing of furin (NM_002569):	
F.1	TRCN0000262166; NM_002569.2-3495s21c1
F.2	TRCN0000262167; NM_002569.2-514s21c1
F.3	TRCN0000262168; NM_002569.2-698s21c1
F.4	TRCN0000075238; NM_002569.2-2954s1c1
F.5	TRCN0000282134; NM_002569.2-1809s21c1
Non-target control used:	MISSION® pLKO.1-puro Non-Mammalian shRNA Control Plasmid DNA

Cell Lysis and Quantitative Immunoblot Analysis

One million cells were lysed in 50 µl lysis buffer consisting of 20 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1% (v/v) Triton X-100, 1 mM EGTA, 2.5 mM Sodium-Pyrophosphate Decahydrate and 1 mM β-glycerophosphate supplemented

with complete protease inhibitors (Roche). Where indicated, samples were digested for 1-3 hrs at 37 °C with PNGAse F (NEB). Proteins were separated by SDS-PAGE, transferred to PVDF membranes, blocked for 1 hr with 5% (wt/v) skim milk in PBS with 0.5% (v/v) Tween-20 and were probed for 1 hr with the appropriate antibodies. Membranes were washed three times with PBS with 0.5% (v/v) Tween-20 and were incubated for 1 h with horseradish peroxidase–conjugated secondary antibody. Proteins were visualized with an enhanced chemiluminescence detection reagent (Pierce). For quantitative analysis, luminescence of Western Blots was captured with the ChemiDoc™ MP system (Bio-Rad Laboratories) and band intensity was analyzed using ImageJ software (NIH, downloaded from: http://rsb.info.nih.gov/ij/index.html) and Microsoft Excel. To calculate the percentage of truncated TLR7 per lane, the intensity of the band of processed TLR7 was divided by the intensity of the band of total TLR7 (shorter + longer fragment).

Large-scale Immunoprecipitation and Tandem Mass Spectrometry

PMA-differentiated cells were lysed as described above. Cell lysate was pre-cleared for 1 hr at 4 °C with mouse IgG-Agarose (Sigma). For immunoprecipitation, samples were incubated for 2.5 hrs at 4 °C with anti-HA-Agarose Clone HA-7 (Sigma). Agarose was washed four times with ice-cold NET buffer (50 mM Tris-Cl pH 7.5, 5 mM EDTA, 150 mM NaCl and 0.5 % NP-4), and bound proteins were eluted by adding Elution Buffer (100 mM Glycine, pH 2.5) and incubating on a rotator at 4 °C for 20 min. Eluted proteins were precipitated using chloroform-methanol precipitation (Wessel and Flugge, 1984). Proteins were dissolved by boiling in SDS sample buffer, separated by 8 % SDS-PAGE, and polypeptides were visualized by silver staining. Bands of interest were excised, digested in-gel with Trypsin, and were analyzed by nano liquid chromatography electrospray ionization tandem mass spectrometry (nLC-ESI-MS/MS) as described previously (Batycka et al., 2006; Mackeen et al., 2010).

Intracellular staining for IL-8

PMA differentiated THP-1 cells were stimulated with 10 µg/ml R837. 1 hr after stimulation Golgi-Plug (BD Bioscience) was added for ≤ 12 hrs, and cells were then fixed, permeabilized and stained with anti-IL8-PE or corresponding isotype control according to the manufacturer's protocol (eBioscience).

Semi-quantitative RT-PCR

Total RNA was isolated from human cells using RNeasy kit from Qiagen. 2 µg of RNA were reverse transcribed into cDNA with SuperScript III Reverse transcriptase (Invitrogen) using Oligo(dT)12-18 primers, according to the protocol of the manufacturer. To detect expression of prosegments of furin-like PCs, and GAPDH as housekeeping gene, transcripts were amplified by PCR using the following sense and antisense primers, respectively, to amplify ppPC5/6 (5′-GAACCGCTGCAGCCG-3′ and 5′-TGTGGGTTCCTCTGCTCG-3′), ppPC7 (5′-ACAGAAAGTCCCACGCTTG-3′ and 5′-CTCTTCCTTCCTCTCACCTTC-3′), and human GAPDH (5′-CCAGCCGAGCCATCGCTC-3′ and 5′-ATGAGCCCCAGCCTTCTCCAT-3′). One twentieth of cDNA was diluted 1:20 in RNase free water and 20 µl cDNA were added to 30 µl of the reaction mix with gene specific primers dependent on the fragment to be amplified. The reaction mix was set up as follows:

Template	20 µl diluted cDNA
MgCl2	1.25 µl
10× PCR buffer	5 µl
Forward primer (5µM)	5 µl
Reverse primer (5µM)	5 µl
dNTP Mix (10 mM each)	1 µl
Taq Polymerase	0.025 U
Water	add to 50 µl

Amplification was conducted using following temperature program:

Step	Temperature	Time	No. of cycles
Initial Denaturation	94 ºC	3 min	1
Denaturation	94 ºC	0.5 min	
Annealing	57 ºC	0.5 min	40
Elongation	72 ºC	1 min	
Final Elongation	72 ºC	5 min	5
	hold at 4 ºC		

Finally, PCR products were analyzed by electrophoresis on agarose gel and photographed.

Quantitative real-time PCR (qPCR)

RNA was prepared from 10^6 cells using Nucleospin RNA II kit (Macherey-Nagel) following the manufacture's instruction. cDNA was prepared from 1µg RNA (measured by NanoDrop) using Retroscript Kit from Ambion (Invitrogen). 1/20th of the cDNA was used in a TacMan qPCR with Universal PCR Master mix and TaqMan Gene expression assays (both from Applied Biosystems). TaqMan gene expression assays used were as follows: for Furin: HS00965485_g1 and HS00159829_m1, for PC5: HS00196400_m1, for PC7: HS00237114_m1, for HPRT1: 4333768, for Actin: 433762, and for GAPDH: HS99999905_m1. Fold-changes in expression relative to control untreated samples were calculated according to the $2-\Delta\Delta CT$ method. Data were processed and analyzed using Microsoft Excel and GraphPad Prism Version 5.0d (GraphPad Software Inc).

Confocal microscopy

THP-1 cells were stimulated with PMA, harvested, and cultured on poly-L-lysine coated glass slides for 1 hr or 24 hrs at 37 °C. Samples were fixed with 4 % paraformaldehyde (Sigma) for 15 minutes at room temperature and permeabilized twice for one minute with 0.5 % Triton-X 100. Blocking buffer (PBS containing 5 % human serum, PAA; 5 % FCS, and 1 % BSA,) was added for 2 hrs before samples

were treated with primary antibodies in blocking buffer for 1 hr. Samples were washed three times with PBS and stained with secondary antibodies in blocking buffer for 1 hr, washed again as before, and then mounted using fluoromount containing DAPI (Southern Biotech). All images were taken to avoid saturation using a confocal microscope (either Zeiss 780 or Zeiss 510; Carl Zeiss), and analysis was done on raw image data. For quantitative co-localization analysis Li´s coefficient (Li et al., 2004) was calculated using the application Coloc2 in Fiji (Rasband, 1997-2012) (http://imagej.nih.gov/ij/) using a 20-fold background filter. Such analysis allows characterizing the degree of overlap between two channels in the multidimensional microscopy image recorded at different emission wavelengths. Images where then adjusted to emphasize the results of the analysis.

Supplemental References

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