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Supplementary Information for

Cathelicidin and PMB neutralize endotoxins by multifactorial mechanisms including LPS-interaction and targeting of host cell membranes

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SUPPLEMENTARY INFORMATION TEXT

Supplementary Materials and Methods

Reagents

The peptides were labeled with small fluorophores as follows. Lissamine rhodamine B or N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) was conjugated to the N-termini of synthesized LL-32 and L-Pep19-2.5 (LL-32-Rh, LL-32-NBD, Pep19-2.5-NBD). PMB (Life Technologies, Thermo Fisher Scientific), a nearly three-fold smaller cyclic lipopeptide, was conjugated to 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene (BODIPY) using a newly developed protocol in which BODIPY® FL-C_5 NHS Ester (Molecular Probes, Thermo Fisher Scientific) was coupled to the free amino groups of the peptide with a C_5 carbon spacer between to yield a green fluorescent peptide (PMB-BODIPY). A mixture of mono- and di-substituted fluorescent peptides was purified by HPLC. The peptide quality was assessed by HPLC and a mass spectrometry analysis.

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), egg chicken L-α-phosphatidylcholine (PC), bovine liver L-α-phosphatidylethanolamine (PE), porcine brain L-α-phosphatidylserine (PS), porcine brain sphingomyelin (SM) and ovine wool cholesterol (Chol) were purchased from Avanti Polar Lipids. Lissamine™ rhodamine B 1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (Rh-DHPE), N-(7-nitrobenz-2-Oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (NBD-PE) and β-BODIPY® FL C5-HPC (PC-BODIPY; 2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-pentanoyl)-1-hexa-decanoyl-sn-glycero-3-phosphocholine) were purchased from Invitrogen and Molecular Probes, respectively.

Macrophage model membranes

The lipid mixture resembling the composition of macrophage membranes (PL_{MAK}) was prepared by mixing the phospholipids from chloroform stocks to a final molar ratio [PC:PS:PE]:SM = 1:0.4:0.7:0.5 M + cholesterol 0.5 M or 0.2 M. For DOPC:SM:Chol model membranes lipids were mixed to a final ratio of 9:9:2 M or 2:2:1 M as indicated for the respective experiments. The organic solvent was evaporated under a stream of nitrogen until completely dry. Lipids were suspended in 20 mM HEPES, 150 mM NaCl, pH 7.4 to a final concentration of 1 mM. Liposome formation was induced by pulsed ultrasound (Ultrasonic-Homogenizer *HTU Soni130*, 1 min, pulse on/off: 2 s, amplitude 30%) followed by three rounds of thermocycling between 4°C and 56°C for 30 min each. Preparations were stored overnight at 4°C before use.

Stimulation of human macrophages by LPS

Human mononuclear cells (MNC) from anonymous healthy donors were isolated from heparinized peripheral blood using the Hypaque–Ficoll gradient method. The experimental

use of MNC was approved by the Ethical Commission of the University of Lübeck (12-202A). All volunteer donors provided informed consent prior to the procedure. Collected MNCs were harvested, washed, and cultivated for 7 days in Teflon bags containing RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 4% heat-inactivated human AB serum, and 2 ng/ml human M-CSF for differentiating monocytes to macrophages. The cultures were incubated at 37°C in a 5% CO_2 atmosphere.

For stimulation experiments, macrophages were suspended in RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 4% human AB serum (complete medium) and seeded into 96-well tissue culture plates at a density of 10^5 cells/well. The cells were incubated with peptides at the indicated concentrations for 30 min at 37°C and were subsequently washed three times to remove non cell-bound peptide or stimulated directly with LPS for 4 h at 37°C. Cell-free supernatants were collected and analyzed in duplicate using an OptEIA set to determine the concentration of human TNF-α (BD Biosciences). The reported data are representative of at least three independent experiments involving cells from different donors.

To detect intracellular TNF-α protein, macrophages were seeded into 5 ml Falcon tubes in RPMI 1640 complete medium containing 10 µg/ml bafilomycin to prevent protein secretion. The cells were incubated with LL-32 or PMB for 30 min at 37°C and subsequently stimulated with LPS or the cytokine IL-1 (PeproTech) at the indicated concentrations. After 4 hours, the cells were washed twice in ice-cold PBS, permeabilized in 0.1% SAP-buffer and stained with a fluorescein-conjugated antibody specific for human TNF-α (R&D Systems). A flow cytometry analysis of the cells was performed on a FACSCalibur system (BD Biosciences) using BD CellQuest software, version 6.0 (BD Biosciences). Figure S1 depicts the strategy used to gate macrophages in the MNC population. The data analysis was performed using WinMDI software (Scripps Research Institute). In each experiment, paired samples were stimulated in the absence of bafilomycin, and the concentration of secreted TNF-α protein in the supernatant was measured by ELISA. The published flow cytometry data presented are representative of three independent experiments performed using cells from different donors.

Cell lines

The HEK293-TLR4/MD-2 cell line was described earlier (1) and maintained in DMEM medium (Biochrom) containing 10% low-endotoxin-grade fetal calf serum (Linaris), 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine in the presence of 400 U/ml hygromycin and 0.5 mg/ml G418. Wildtype HEK293 cells were maintained in DMEM medium containing 10% low-endotoxin-grade fetal calf serum (Linaris), 100 U/ml penicillin, 100 µg/ml streptomycin, without selection antibiotics. The culture was maintained at 37°C in an atmosphere of 5% CO_2. For experiments, HEK293-TLR4/MD-2 cells in DMEM medium containing 10% fetal calf serum (FCS) were seeded into 96-well

plates at a density of 5×10^4 cells/well and allowed to adhere for 1 h. The peptides were diluted in 20 mM HEPES buffer (pH 7.4) and added to the wells at the indicated concentrations. After a 30 min incubation at 37°C, the cells were washed three times to remove free peptide or stimulated directly with LPS, IL-1β, or TNF-α (PeproTec) for 24 h at 37°C. For cholesterol-depletion experiments, the wells of 96-well culture plates were treated with *β*-methyl-cyclodextrin (Sigma-Aldrich) in serum-free DMEM for 1 h at 37°C. After washing, fresh DMEM containing 10% FCS was added to the plates, and the cells were stimulated with LPS as indicated. Cell-free supernatants were collected, and the concentrations of human IL-8 were analyzed in duplicate using an OptEIA set (BD Biosciences). All experiments were performed in triplicate, and the data represent the means and ±SEM of at least three independent experiments.
CHO cells (ATTC #CCL-61) that had been stably transfected with mGFP-GPI (please refer to (2) for details) were grown in DMEM/F12 medium (PAA-Laboratories) supplemented with 10% fetal calf serum (PAA-Laboratories) and 400 µg/ml G418 (PAA-Laboratories). The cells were cultured on 10 cm tissue culture plates (Greiner Bio-one) in a humidified atmosphere at 37°C and 5% CO_2. For experiments, the cells were harvested using Accutase (eBioscience), seeded into eight-well Lab-Tek chambered slides (Nunc), and allowed to reach 50% confluency on the day before measurements. Before peptide incubation, the cells were rinsed twice with HBSS containing calcium and magnesium (PAA-Laboratories). All experiments were performed at 37°C with peptides remaining in solution.

Quantitative real-time PCR (qRT-PCR)
Human macrophages were seeded in 96-well plates at a density of 5×10^5 cells/well; incubated with PMB, LL-32, or control medium for 30 min at 37°C; and subsequently stimulated with LPS. After 1 h of stimulation, the cells in each well were harvested with 200 µl FCP-buffer from the FastLane cDNA kit (Qiagen) for RNA isolation. To generate cDNA, total RNA was isolated from the cell lysates and reverse-transcribed using the FastLane cDNA kit (Qiagen). Gene-specific primer pairs and Universal Probe Library probes (see Table S2) were obtained from Roche Diagnostics and used in a TaqMan assay. Quantitative real-time PCR amplification was performed on a LightCycler 480 II system (Roche Diagnostics). The threshold values (C_t values) were determined using LightCycler 480 software, and the relative expression ratios of the target gene to the reference gene (HPRT) and the normalization of samples to the untreated control were calculated according to the $\Delta\Delta C_t$ method. The data represent the results of three independent experiments using cells from different donors.

Confocal microscopic analysis of giant unilamellar vesicles (GUVs)
Giant unilamellar vesicles (GUVs) were prepared by electroformation as described elsewhere (3). GUVs were reconstituted from DOPC:SM:Chol at a lipid ratio of 2:2:1 M.

Fluorescently labeled and biotinylated lipids were dissolved directly in ethanol (p.a.) to final concentrations of 0.5 and 2 mg/ml. Finally, GUVs were electroformed in 10 mM sucrose at 55°C for 5 h (3 V, 10 Hz) and cooled to room temperature overnight.
The supported biotinylated bilayer (SBB): DOPC was prepared by dissolution in $CHCl_3$ to a final concentration of 2 mg/ml and was mixed with biotinylated PE in a ratio of 99.5:0.5 mol%. The lipid mixture was evaporated under nitrogen, and the resulting lipid film was resolved in 5 mM HEPES (pH 7.4) to a final concentration of 1 mg/ml. Vesicles were formed by the application of ultrasound pulses (Ultrasonic-Homogenizer *HTU Soni130*, 2 min, pulse on/off: 2 s, amplitude 80%). The vesicle solution was pipetted directly into an eight-well microscopy chamber (LabTekII®, Thermo Fisher Scientific; 150 µl/well). Biotinylated vesicles were spread during overnight incubation at 4°C on a stirring plate (70 rpm).
Immobilization of GUVs (iGUVs) was achieved by linking the vesicles to the SBB using avidin–biotin chemistry. The GUVs were added to the SBB at a ratio of 3:1 (v/v) and linked by the addition of 15 µl avidin (1 mg/ml in MilliQ), followed by a 30 min incubation. Fluorescence dye distribution was detected using a Leica TCS SP5 confocal laser scanning microscope (Leica Microsystems) equipped with Leica LAS AF software. For further processing, the user procedure *GUV-analysis.py* [Python(x, y), Version 2.7.6.1] was used to determine the fluorescent-dye-distribution-analysis (FDDA) of each channel. The program code for the FDDA is provided under SI program code.

Single-molecule fluorescence microscopy

TOCCSL ("Thinning Out Clusters while Conserving Stoichiometry of Labeling"), a single-molecule fluorescence modality (2, 4), was used to evaluate the mGFP-GPI homo-association on the plasma membranes of living CHO cells. Briefly, an Axiovert 200 microscope equipped with a 100x Plan-Apochromat objective (NA = 1.46; Zeiss) was used to illuminate samples in an objective-based total internal reflection (TIR) configuration via the epiport. Illumination at 488 nm was provided by an Ar^+ laser (Model 2017-05AR, Spectra Physics) with a typical power of 2–11 kW/cm² on the sample. A slit aperture (Zeiss) with an approximate width of 7 µm in the object plane was used as a field stop to confine the area of illumination. To ensure exact timing, the excitation path was equipped with an acousto-optic modulator (Isomet) and a mechanical shutter (Vincent Associated). Timing protocols were generated using in-house programs implemented in Labview and were executed using a high-speed analog output card (National Instruments). The emission light was filtered (HQ535/50 and 505DCLP, Chroma), and fluorescence images were recorded using a back-illuminated, nitrogen-cooled CCD camera (ln/CCD-1340/1300-eb/1, Roper Scientific). To ensure precise temperature control, an in-house incubator equipped with a heating unit and an objective heater (PeCon) were used. All experiments were performed at 37°C.

After recording a pre-bleach image at a power density of 2 kW/cm² and an illumination time of 1 ms, the samples were bleached at a power density of 11 kW/cm² for 200–450 ms. The efficiency of photobleaching was tested by recording an image 1 ms after the bleach pulse. After a recovery period of 600–2400 ms, sequences of up to 10 images at a delay of 20 ms were recorded using the same illumination settings reported for the pre-bleach image. The first image after recovery was used to analyze the brightness of individual mGFP-GPI homo-associates, while the last image of the sequence was used to determine the reference brightness of a single mGFP molecule. Because only a small area of the cell was photobleached, multiple bleach- and recovery runs could be performed on a single cell. For the analysis, single-molecule signals were analyzed using in-house algorithms implemented in MATLAB (MathWorks). The position, integrated brightness *B*, full width at half maximum, and local background of each signal was determined. The *B* values of single mGFP-GPI molecules were pooled from the final images of all TOCCSL sequences and used to calculate the probability density function (pdf) of the monomers as $\rho_1(B)$. The independent photon emission process enabled the calculation of the corresponding pdfs of *N* co-localized emitters by a series of convolution integrals, $\rho_N(B) = \int \rho_1(B')\rho_{N-1}(B - B')dB'$. A weighted linear combination of these pdfs was then used to calculate the distribution of brightness in a mixed population of monomers and higher-order multimers, $\rho(B) = \sum_{N=1}^{N_{max}} \alpha_N \rho_N(B)$. The brightness values from all TOCCSL images of multiple cells per experimental condition were used to calculate $\rho(B)$. A least-square fit was applied to determine the weights of the individual pdfs, α_N, with $\sum_{N=1}^{N_{max}} \alpha_N = 1$. A minimum of 250–500 brightness values were used to calculate $\rho_1(B)$ and $\rho_2(B)$. No higher order multimers were present in samples. The distribution of α_2 values was determined by bootstrapping. The mean and standard deviation (SD) of α_2 were calculated from this distribution and are displayed as error bars. The statistical analysis was performed by comparing the single molecule brightness values of the peptide treatment and the control using a two-sample Kolmogorov-Smirnov test. Testing was done in Matlab using the implemented function kstest2.

Cell viability assays

The cytotoxicity of the tested peptides against human macrophages and HEK293-TLR4/MD-2 cells was determined using an MTT assay. The peptides were diluted from stock solutions in complete cell culture medium and incubated with the cells for 4 h (human macrophages) or 24 h (HEK293 cells) at 37°C. “Incubation time” refers to the incubation time during a stimulation experiment. Cell metabolic activity, a measure of cell viability, was determined via an additional 2 h incubation at 37°C in the presence of 5 mg/ml MTT (Sigma-Aldrich) in PBS. The reaction was terminated with stop-reagent, and the absorbance in each plate well at 570 nm (A_{570}) was analyzed photometrically. The data indicate the metabolic activity as the % viability of the control.

Human erythrocytes isolated from the blood of healthy donors were subjected to a hemolysis assay. Erythrocytes in PBS (pH 7.4) were seeded in 96-well round-bottomed plates at an $OD_{412\ nm}$ of 1.4. Peptides were diluted in PBS, added to the cells, and the cells were incubated for 30 min at 37°C. Hemolysis was determined according to the A_{405}. Hemolysis was calculated as a percentage of the control (Triton-X 100-lysed erythrocytes). Data represent the mean and ± SEM of three independent experiments performed in duplicate.

X-ray reflectivity (XRR) measurements

Solid supported membrane stacks of were prepared on silicon-(111)-wafers (dimensions of 10x15 mm^2) with a thickness of 500 µM (Silchem, Freiberg, Germany). Si-wafers were cleaned by subsequent and repeated sonication in MeOH and ultrapure water (three times, each step for 10 min). Right before sample deposition, wafers were plasma cleaned (air plasma, 2.5 min; PDC-002, Harrick Plasma, Ithaca, NY, USA). DOPC:SM:Chol (2:2:1 M) dissolved in $CHCl_3$ (10 mg/mL) were either applied pure or mixed with LL-32 or PMB (4:1; v/v) on the Si-wafers. For full evaporation of the solvent, samples were dried overnight. Experiments were carried out at the synchrotron beamline P08 of PETRAIII (DESY, Hamburg) at a nominal humidity of 98% rH and a photon energy of 25 keV. The X-ray beam was collimated to a size of 150x500 µm² (v x h). Reflectivity profiles were acquired with an angular resolution of 0.01° and an acquisition time of 1 s for each position. XRR-data of solid supported membrane stacks were first evaluated with OriginPro® 8 (OriginLab Corporation, Northhampton, MA, USA) to obtain the electron density distribution. General proceedings include background and baseline corrections. Bragg peaks were fitted with a Gaussian or Lorentz fit.

Calorimetric analysis

Calorimetric effects of the binding of PMB to DOPC:SM:Chol liposome membranes were analyzed by differential scanning calorimetry. Calorimetry measurements were performed with a VP-DSC calorimeters (MicroCal, Inc., Northampton, MA, USA) at a heating and cooling rate of 1 $K \cdot min^{-1}$. The accuracy of the DSC experiments was $\Delta T = 0.1$ °C for the main phase transition temperatures. The measurements of DOPC:SM:Chol (9:9:2 M) small unilamellar liposomes at 10 mM in 20 mM HEPES, 150 mM NaCl, pH 7.4 were performed in the temperature interval from 5°C to 95°C. For each condition, five consecutive heating and cooling scans were performed to analyse the reproducibility of the DSC experiment. The DSC data were analysed using the Origin software. In the figure, only the temperature range at which phase transitions were observed is shown.

SUPPLEMENTAL FIGURES

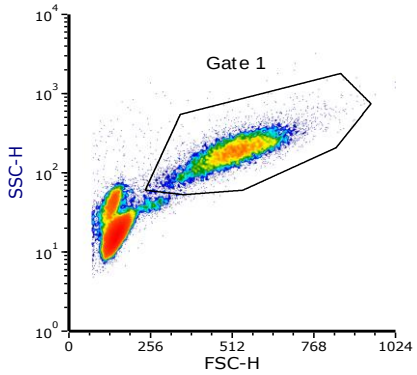

Figure S1: **Gating strategy for the flow cytometry analysis of human macrophages**

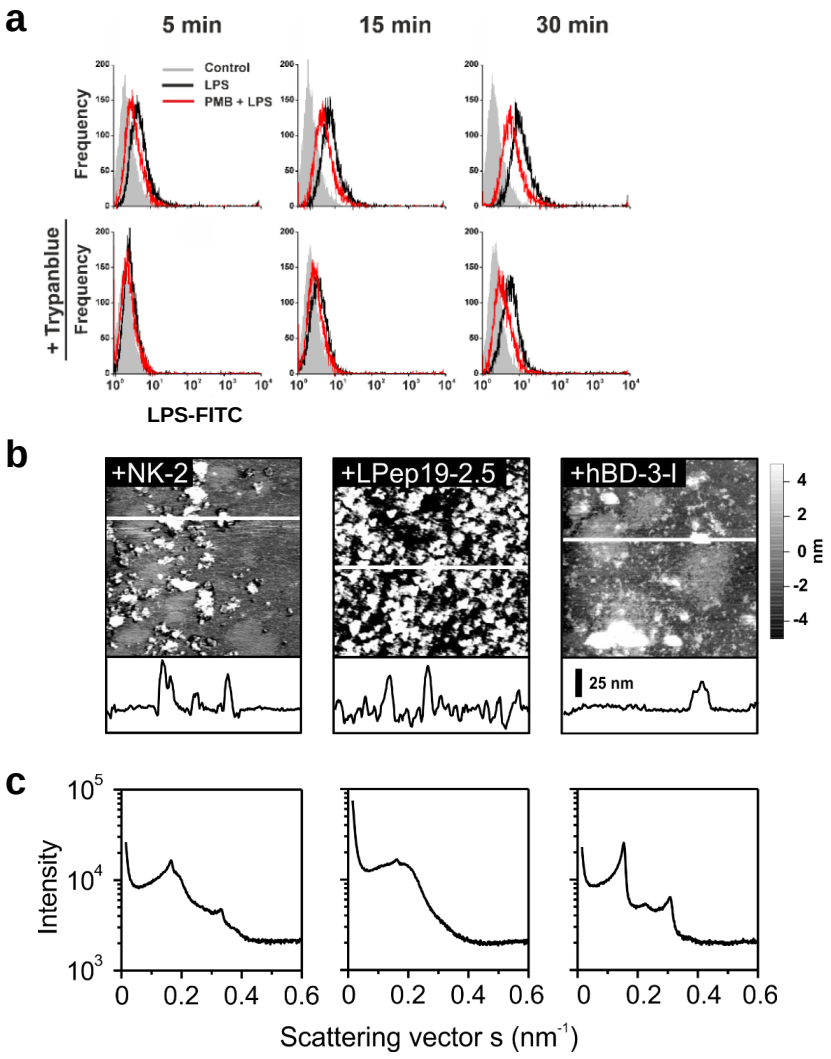

Figure S2: PMB reduces the cellular binding of lipopolysaccharide (LPS)
(a) Macrophages were seeded at a density of 10^5 cells and stimulated with 3 µM LPS-FITC in the presence of 3 µM PMB for 5, 15 and 30 min at 37°C. The cells were washed, fixed in 2% paraformaldehyde, and analyzed by flow cytometry. The data are representative of n = 5 independent experiments.
(b) Atomic force microscopy (AFM) images and height profiles of solid-supported layers of LPS WBB01. LPS was immobilized as the control, or preincubated with peptide at a ratio of 2:1 (by weight). Data are representative of n = 3 independent experiments.
(c) Small-angle X-ray scattering (SAXS) diffractograms of pure LPS aggregates (left panel) and aggregates prepared in the presence of LL-32 (middle panel) or PMB (right panel) at a LPS:peptide ratio of 2:1 (by weight). The diffractograms are representative of n = 3 independent experiments.

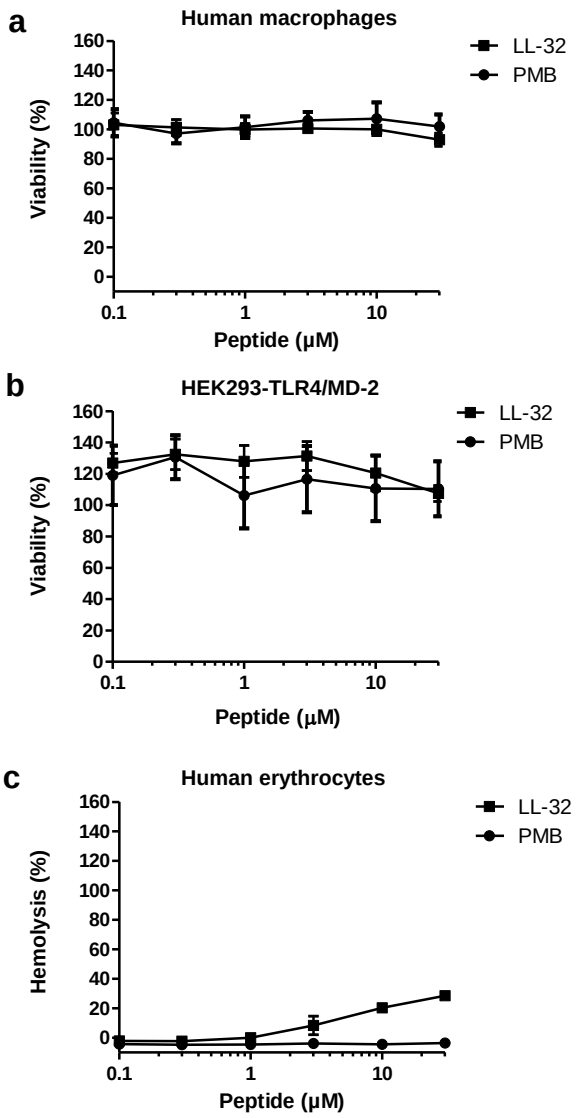

Figure S3: Cytotoxicity of antimicrobial peptides (AMPs)

(**a**) Human monocyte-derived macrophages in complete medium containing 4% human AB serum were seeded in 96-well dishes at a density of 5×10^5 cells per well. The cells were incubated with peptides for 4 h at 37°C as described for the lipopolysaccharide (LPS) stimulation experiments. Cell viability was determined using an MTT assay. The data represent the means ± standard errors of the means (SEM) of n = 4 independent experiments using cells from different donors.

(**b**) HEK293-TLR4/MD-2 cells in DMEM containing 10% fetal calf serum were seeded in 96-well dishes at a density of 5×10^5 cells/ml, allowed to adhere for 1 h at 37°C, and incubated with peptides for 24 h at 37°C as described for the LPS stimulation experiments. Cell viability was determined using an MTT assay. The data represent the means ± SEM of n = 4 independent experiments.

(**c**) Erythrocytes were incubated with peptides for 24 h at 37°C. Hemolysis was determined by measuring the release of hemoglobin. The data represent the means ± SEM of n = 3 independent experiments.

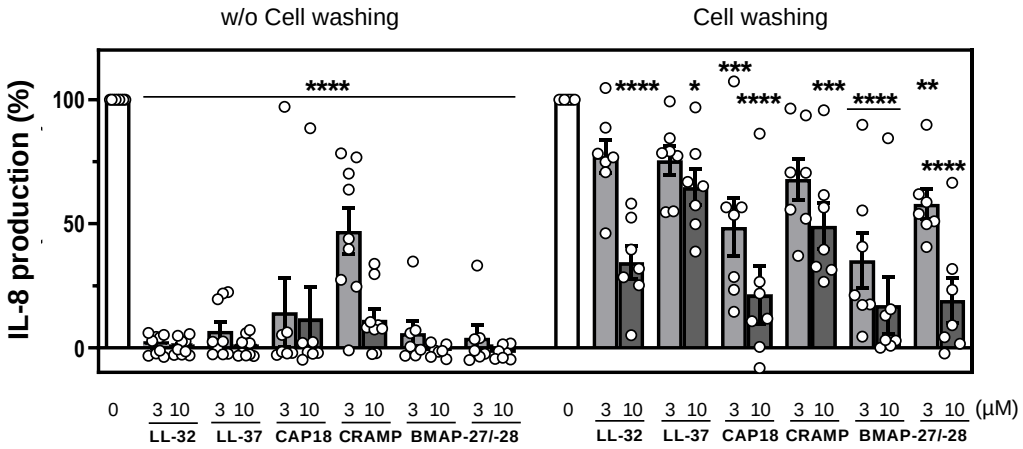

Figure S4: Effects of cathelicidins from different species on cell activation
HEK293-TLR4/MD-cells in DMEM containing 10% fetal calf serum were seeded at a density of 5×10^5 cell/ml, allowed to adhere for 1 h at 37°C and treated with 3 or 10 µM of a representative cathelicidin for 30 min at 37°C. Subsequently, the cells were either stimulated directly with 10 nM lipopolysaccharide (LPS) for 24 h without washing (left) or after three washes to remove unbound peptide (right). LL-32, LL-37, CAP18, CRAMP, BMAP-27, and BMAP-28 were selected as representative cathelicidins. The data are plotted as the means ± SEM of n = 6 - 8 independent experiments. White dots represent the individual data points. The concentrations of secreted IL-8 were determined in cell-free supernatants by ELISA. IL-8 values of cells stimulated with LPS in the absence of peptide were set 100% and all other values were calculated accordingly. The statistical analysis was performed using a one-way ANOVA and Dunnett's post test; *$p \leq 0.05$, **$p \leq 0.01$, and ***$p \leq 0.001$ (peptide groups versus LPS control).

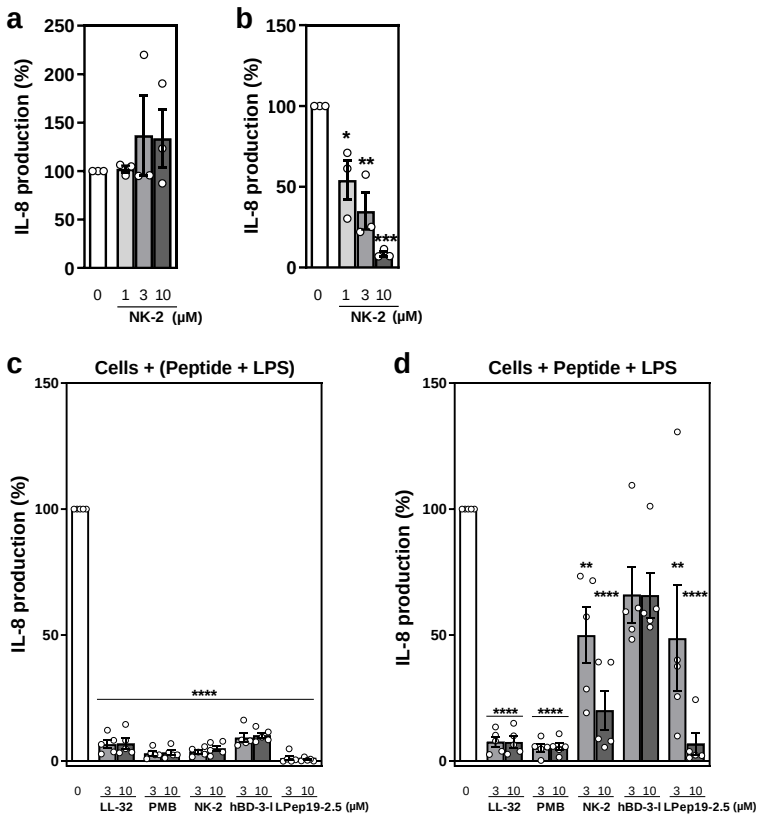

Figure S5: Differential effects of peptides on cell activation

(**a, b**) HEK293-TLR4/MD-2 cells in DMEM containing 10% fetal calf serum (FCS) were seeded at a density of 5×10^5 cell/ml, allowed to adhere for 1 h at 37°C, and treated with 1, 3, or 10 µM NK-2 for 30 min at 37°C. Subsequently, the cells were stimulated with 10 nM lipopolysaccharide (LPS) either (**a**) after three washes to remove unbound peptide or (**b**) directly without a washing step. The concentrations of secreted IL-8 in the cell-free supernatants were determined by ELISA. IL-8 values of cells stimulated with LPS in the absence of peptide were set 100% and all other values were calculated accordingly. The data are shown as the means ± SEM of n = 3 independent experiments. White dots represent the individual data points.

(**c, d**) HEK293-TLR4/MD-2 cells in DMEM containing 1% FCS were seeded at a density of 5×10^5 cell/ml and treated with LPS and peptides in two different preparation sequences. (**c**) LPS was pre-incubated for 30 min at 37°C with the peptides, after which the mixture was added to the cells, or (**d**) the cells were pre-incubated with the peptides for 30 min at 37°C, followed by the addition of LPS. The cells were then incubated for 24 h at 37°C. The concentrations of secreted IL-8 in the cell-free supernatants were determined by ELISA. IL-8 values of cells stimulated with LPS in the absence of peptide were set 100% and all other values were calculated accordingly. The data are reported as the means ± SEM of n = 5 independent experiments.

The statistical analysis was performed using a one-way ANOVA and Dunnett's post test; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$ (peptide groups versus LPS control).

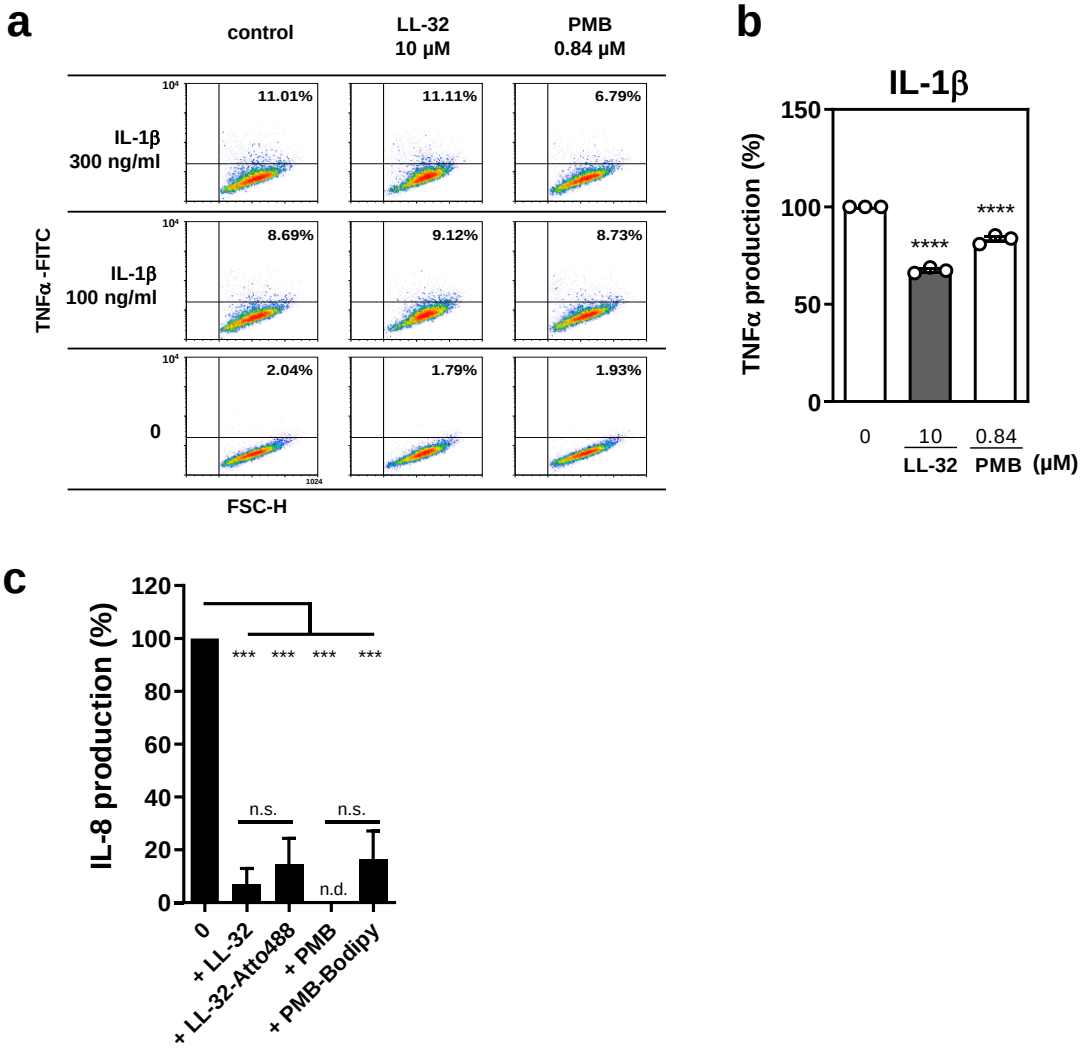

Figure S6: Effects of peptides on IL-1β-stimulated human macrophages

(**a**) Macrophages were pre-incubated with LL-32 (3, 10 µM), PMB (1 µg/ml, 0.84 µM), or buffer (control) for 30 min at 37°C and subsequently stimulated with 100 or 300 ng/ml IL-1β for 4 h in the presence of 10 µg/ml bafilomycin to prevent cytokine secretion. The cells were fixed and permeabilized by Triton X-100, and intracellular TNF-α protein was labeled with a specific antibody prior to a flow cytometry analysis. The numbers in the upper right quadrants represent the percentages of gated macrophages positive for TNF-α. The data are representative of n = 3 independent experiments.

(**b**) Human macrophages were incubated with LL-32 (10 µM), PMB (1 µg/ml, 0.84 µM), or buffer (control) for 30 min at 37°C and subsequently stimulated with 300 ng/ml IL-1β for 4h. The concentrations of TNF-α in the cell-free supernatants were analyzed using an ELISA. The data were normalized to the IL-1ß control and are shown as the means ± SEM of n = 3 independent experiments using cells from different donors. White dots represent the individual data points. The statistical analysis was performed using a one-way ANOVA and Dunnett's post test; ****$p \leq 0.0001$ (peptide groups versus IL-1β control).

(**c**) HEK293-TLR4/MD-2 cells were incubated with LL-32, PMB or the fluorophore-conjugated peptides LL-$32_{Atto488}$ and PMB-$_{Bodipy}$ at 1 µM concentration for 30 min at 37°C. Subsequently LPS was added at 50 nM and the cells were incubated for 24h at 37°C. The concentrations of secreted IL-8 in the cell-free supernatants were determined by ELISA. IL-8 values of cells stimulated with LPS in the absence of peptide were set 100% and all other values were calculated accordingly. The data are reported as the means ± SEM of n = 3 independent experiments. The statistical analysis was performed using a one-way ANOVA and Dunnett's post test; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, and ****$p \leq 0.0001$ (peptide groups versus LPS). Inhibitory activity of unlabeled peptide versus fluorophore-labeled peptide were analyzed by two-sided Student's t-Test of paired samples.

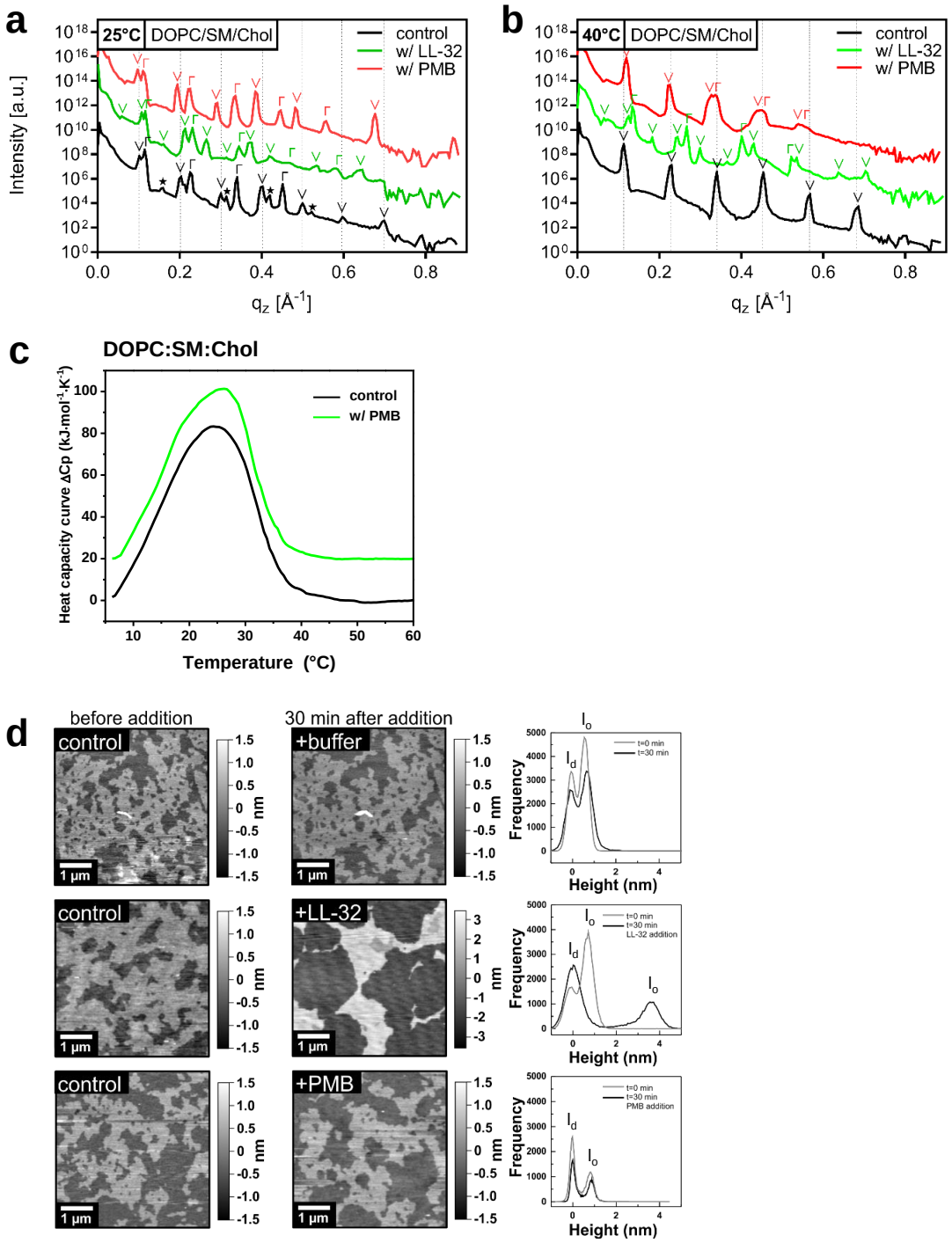

Figure S7: Effects of peptides on membrane organization and phase transition thermodynamics of DOPC:SM:Chol membranes

(a) X-Ray reflectivity (XRR)-profiles of solid-supported multilayers of DOPC:SM:Chol (2:2:1 M) alone and in presence of the peptides LL-32 and PMB (Lipid : peptide 4:1 v/v) at 25°C and (**b**) at 40°C. Control measurement of the pure membrane multilayer (black);

membrane multilayer after exposure to LL-32 (green) and after exposure to PMB (red). For the sake of clear arrangement, the green data sets (LL-32) were shifted by a factor of 10^4 and the red data sets (PMB) by a factor of 10^7. Reflectivity of domains with different membrane bilayer thickness are indicated by V (l_d, liquid disordered) and Γ (l_o, liquid ordered). Peaks that cannot be assigned to either of these domains are marked by *. At 25°C, all XRR profiles indicate a two-phase state. At 40°C, only for the membrane multilayers exposed to LL-32 the two coexisting domains can be assigned.

(**c**) Differential scanning calorimetry performed on DOPC:SM:Chol (9:9:2 M) liposomes at 10 mM in 20 mM HEPES, 150 mM NaCl, pH 7.4. For each condition, five consecutive heating and cooling scans were performed. PMB was added to a final concentration of 25 µM. In the figure, only the temperature range at which phase transitions were observed is shown.

(**d**) Atomic force microscopy (AFM) images and height histograms of the solid-supported bilayers of DOPC:SM:Chol (9:9:2 M). DOPC:SM:Chol bilayers were immobilized on mica and washed. Peptides were added to a final concentration of 25 µM. The presented images were obtained before and 30 minutes after the addition of peptide (or buffer as a control). The data are representative of n = 3 independent experiments.

Table S1: Peptide sequences

Peptide	Origin	Amino acid sequence	MW
LL-32	human	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLV-NH_2	3921.7
LL-37	human	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-NH_2	4492.3
CAP18	rabbit	GLRKRLRKFRNKIKEKLKKIGQKIQGLLPKLAPRTDY-$CONH_2$	4432.5
CRAMP	murine	GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPE-$CONH_2$	3749.5
BMAP-	bovine	GRFKRFRKKFKKLFKKLSPVIPLLHL-$CONH_2$	3225.1
BMAP-	bovine	GGLRSLGRKILRAWKKYGPIIVPIIRI-$CONH_2$	3073.9
hBD-3-l	human	GIINTLQKYYSRVRGGRSAVLSSLPKEEQIGKSSTRGRKSSRRKK	5063.6
NK-2	porcine	KILRGVCKKIMRTFLRRISKDILTGKK-$CONH_2$	3202.0
LPep19-	synthetic	GCKKYRRFRWKFKGKFWFWG-NH_2	2712.2

Table S2: Gene-specific primers used for cDNA amplification during real-time PCR. HPRT, hypoxanthine–phosphoguanine ribosyltransferase; UPL-probe #, universal-probe-library number (Roche Diagnostics).

Target gene	Primer	Sequence (5´–3´)	UPL-probe
HPRT	forward	tga cct tga ttt att ttg cat acc	# 73
	reverse	cga gca aga cgt tca gtc ct	# 73
TNF-α	forward	cag cct ctt ctc ctt cct gat	# 29
	reverse	gcc aga ggg ctg att aga ga	# 29
IL-1β	forward	tac ctg tcc tgc gtg ttg aa	# 78
	reverse	tct ttg ggt aat ttt tgg gat ct	# 78
IL-8	forward	aga cag cag agc aca caa gc	# 72
	reverse	atg gtt cct tcc ggt ggt	# 72

SI Program Code

GUV_analysis.py – Code for FDDA of GUVs [Python(x, y), Version 2.7.6.1]

```
#!/usr/bin/python
# -*- coding: utf-8 -*-

from pylab import *
#import math
matplotlib.rcParams.update({'font.size': 16, 'family': 'sans-serif', 'sans-serif':['Arial']})
#import os
from scipy import misc
import numpy
from extract_profile import *


exp = 1

if exp == 1:
    x0 = 533
    y0 = 546
    r = 346
    dr = 10
    ang0, az_sum0, az_me0 =extract_azimuthal('LP076_221_BODIPY-PMB_Image026 - C=0.png',x0,y0,r,dr)
    ang1, az_sum1, az_me1 =extract_azimuthal('LP076_221_BODIPY-PMB_Image026 - C=1.png',x0,y0,r,dr)


az_sum0 = az_sum0 - 0.0
az_sum1 = az_sum1 - 0.0

#az_sum0 = az_sum0/float(np.max(az_sum0))
#az_sum1 = az_sum1/float(np.max(az_sum1))

#az_sum1 = az_sum1*float(np.max(az_sum0))/float(np.max(az_sum1))


fig, ax = plt.subplots(dpi=150,figsize=(10,3))
ax.plot(ang0,az_sum0,'g',label='PMB')
ax.plot(ang1,az_sum1,'r',label='$L_D$-domain')
ax.set_xlim(0,360)
ax.set_ylim(0,max(np.max(az_sum0),np.max(az_sum1)))#
ax.set_title('Summed Intensity')
ax.set_xlabel('Angles / deg')
ax.set_ylabel('Intensity / a.u.')
handles, labels = ax.get_legend_handles_labels()
lgd = ax.legend(handles, labels, loc='upper right', bbox_to_anchor=(0.1,-0.1))
fig.tight_layout()
plt.show()
fig.savefig('samplefigure_summed', bbox_extra_artists=(lgd,), bbox_inches='tight')


fig, ax = plt.subplots(dpi=150,figsize=(10,3))
ax.plot(ang0,az_me0,'g',label='PMB')
ax.plot(ang1,az_me1,'r',label='$L_D$-domain')
ax.set_xlim(0,360)
ax.set_title('Mean Intensity')
ax.set_xlabel('Angles / deg')
ax.set_ylabel('Intensity / a.u.')
handles, labels = ax.get_legend_handles_labels()
lgd = ax.legend(handles, labels, loc='upper right', bbox_to_anchor=(0.1,-0.1))
fig.tight_layout()
plt.show()
fig.savefig('samplefigure_mean', bbox_extra_artists=(lgd,), bbox_inches='tight')
```

extract_profile.py

```
#!/usr/bin/python
# -*- coding: utf-8 -*-

from pylab import *
#import math
matplotlib.rcParams.update({'font.size': 16, 'font.family': 'serif'})
#import os
from scipy import misc
import numpy



def extract_azimuthal(filename,x0,y0,r,dr):
    global x_vals, y_vals, x_val, y_val, x_val_lo, x_val_up, y_val_lo, y_val_up

    dat = misc.imread(filename)
    print dat.shape

    azimuthal_mean_intensity = []
    azimuthal_sum_intensity  = []
    x_val,y_val          = [],[]
    x_val_lo, y_val_lo   = [],[]
    x_val_up, y_val_up   = [],[]

    index_start = r - dr #x0 + r - dr
    index_end   = r + dr #x0 + r + dr
    ny,nx =dat.shape

    xi = np.linspace(0, nx, nx)
    yi = np.linspace(0, ny, ny)

    print xi[0],xi[-1]
    grid_x,grid_y = meshgrid(xi, yi)
    length_i = abs(r+50)#abs(x0-nx)  # available data length starting at primary beam position

    angles=[]
    for i in range(1,2*360):
       angles.append(i/2.0)
    angles = np.array(angles)
    #print 'Angles:', angles

    for angle in angles:
     # full length:
     x1 = x0 + length_i*np.cos(angle*np.pi/180)
     y1 = y0 - length_i*np.sin(angle*np.pi/180)

     # Define the lines
     x, y = np.linspace(x0, x1, length_i), np.linspace(y0, y1, length_i)

     # Extract the values along the lines
     intensity = dat[y.astype(np.int), x.astype(np.int)]
     #absr =   absolutr[y.astype(np.int), x.astype(np.int)]
     x_vals =   grid_x[y.astype(np.int), x.astype(np.int)] # x-values needed for plot only
     y_vals =   grid_y[y.astype(np.int), x.astype(np.int)] # y-values needed for plot only
     azimuthal_mean_intensity.append(np.mean(intensity[index_start:index_end]))
     azimuthal_sum_intensity.append(np.sum(intensity[index_start:index_end]))
     x_val.append(x_vals[r]),         y_val.append(y_vals[r])
     x_val_lo.append(x_vals[index_start]), y_val_lo.append(y_vals[index_start])
     x_val_up.append(x_vals[index_end]),   y_val_up.append(y_vals[index_end])

    i_azi_mean = np.array(azimuthal_mean_intensity)
    i_azi_sum = np.array(azimuthal_sum_intensity)
    fig, axes = plt.subplots(nrows=2)
    axes[0].imshow(dat)
    axes[0].plot(x0,y0, 'go')
    axes[0].plot(x_val_up,y_val_up, 'g.',markersize=3)
    axes[0].plot(x_val_lo,y_val_lo, 'g.',markersize=3)
    #axes[0].plot(x_val,y_val, 'r.')
    axes[0].axis('off')
```

```
    axes[0].axis('image')
    axes[1].plot(angles,i_azi_mean,'k',label='Mean Intensity')
    #axes[1].plot(angles,i_azi_sum,'g',label='Summed Intensity')
    axes[1].set_xlim(0,360)
    axes[1].set_xlabel('Angles / deg')
    axes[1].set_ylabel('Intensity / a.u.')
    #axes[1].legend()
   #axes[1].set_ylim(1000,50000)
    plt.show()


    plt.imshow(dat)
    plt.plot(x0,y0, 'go')
    plt.plot(x_val_up,y_val_up, 'g.',markersize=3)
    plt.plot(x_val_lo,y_val_lo, 'g.',markersize=3)
    plt.axis('image')
    plt.axis('off')
    plt.show()

    fout = open(filename[:-4]+'_'+str(x0)+'_'+str(y0)+'_'+str(r)+'_'+str(dr)+".txt","w") # data storage
    fout.write('point of origin: x0 ='+str(x0)+', y0 ='+str(y0)+" \n")
    fout.write('radius ='+str(r)+'+/-'+str(dr)+" (pixel) \n \n")
    fout.write('angle/degree'+"  "+'SummedIntensity/a.u.'+"  "+'MeanIntensity/a.u.'" \n")
    for i in range(len(angles)):
        fout.write(str(format(angles[i], '.2f'))+"            "+str(format(i_azi_sum[i],'.2f'))+"
"+str(format(i_azi_mean[i],'.2f'))+" \n")
    fout.close()

    azimuthal_mean_intensity = np.float_(np.array(azimuthal_mean_intensity))
    azimuthal_sum_intensity  = np.float_(np.array(azimuthal_sum_intensity))
    return angles, azimuthal_sum_intensity,  azimuthal_mean_intensity

#x0 = 404
#y0 = 429
#r = 114
#dr = 5
#ang, az_sum, az_me =extract_azimuthal('01_A2_DOPC-SM-Chol_221.lif - Image026 - C=0.png',x0,y0,r,dr)
```

SI References

1. S. P. Keese, K. Brandenburg, M. Roessle, & A. B. Schromm, Pulmonary surfactant protein A-induced changes in the molecular conformation of bacterial deep-rough LPS lead to reduced activity on human macrophages. *Innate Immunity* **20**, 787-798 (2014).
2. M. Brameshuber, *et al.*, Imaging of mobile long-lived nanoplatforms in the live cell plasma membrane. *J. Biol. Chem.* **285**, 41765-41771 (2010).
3. S. L. Veatch, Electro-formation and fluorescence microscopy of giant vesicles with coexisting liquid phases. *Methods Mol. Biol.* **398**, 59-72 (2007).
4. M. Moertelmaier, M. Brameshuber, M. Linimeier, G. J. Schütz, & H. Stockinger, Thinning out clusters while conserving stoichiometry of labeling. *Appl. Phys. Lett.* **87**, 263903 (2005).