Cell

Supplemental Information

A Conserved Circular Network of Coregulated Lipids Modulates Innate Immune Responses

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Supplemental Experimental Procedures

Cell culture

RAW264.7 macrophages and human embryonic kidney (HEK293T) cells were cultured in DMEM (Sigma Aldrich) and 10% FCS (Gibco) containing 1% Penicillin-Streptomycin (GE Healthcare) at 37°C and 5% CO_2. The "Cell line and DNA biobank from patients affected by genetic diseases" (Istituto G. Gaslini), member of the Telethon Network of Genetic Biobanks (project no. GTB12001) funded by Telethon Italy, provided us with specimens of human fibroblasts. The following fibroblast samples were obtained from the NIGMS Human Genetic Cell Repository at the Coriell Institute for Medical Research: (GM02075, GM02315, GM05659). Fibroblasts were cultured in DMEM (Sigma Aldrich) and 20% FCS (Gibco) containing 1% Penicillin-Streptomycin (GE Healthcare) at 37°C and 5% CO_2.

Reagents

TLR ligands such as ultrapure lipopolysaccharide (LPS) *E.coli* 0111:B4, Imiquimod (IMQ) – R837, and CpG DNA ODN 1826 were obtained from Invivogen. Interferon-stimulatory DNA (ISD) oligonucleotides (Stetson and Medzhitov, 2006) were synthesized by Microsynth. Poly(dA:dT) was obtained from Sigma Aldrich and Lipofectamine 2000 was obtained from Invitrogen. Cell viability was determined by CellTiter-Glo (Promega) according to the manufacturer's instructions.

TLR stimulation

5×10^5 RAW264.7 cells/ml or human fibroblasts were seeded in 96-well microtiter plates and incubated in serum-free medium for 2 hours prior stimulation. RAW cells were stimulated with the following TLR ligands for indicated time points: LPS (100ng/ml), CpG DNA (5μM), Imiquimod (5μM). For stimulation with ISD (1μg/ml) or poly(dA:dT) (500ng/ml) cells were transfected using Lipofectamine 2000 according to manufacturer's instructions. Human fibroblasts were stimulated with LPS (1μg/ml) or IMQ (25μM). Each stimulation condition was performed in quadruplicates.

shRNA transduction

Briefly, shRNA constructs were obtained as glycerol stocks from Sigma Aldrich and Open Biosystems Thermo Scientific. Lentiviral particles were produced by transfecting helper plasmids together with the pLKO.1 vector containing the shRNA template into 5×10^5 HEK293T. The supernatant was collected for three consecutive days before it was ultracentrifuged at 30,000 rpm for 90 min to concentrate lentiviral particles. Prior to infection, 60-80% confluent 10^6 RAW264.7 cells were treated with polybrene (8µg/ml; Santa Cruz Biotechnology). Then, the produced virus was added in 400µl Optimem (Gibco). After 12 hours the medium was changed and after another 12 hours the culturing medium was replaced by DMEM 10% FCS 1% Penicillin-Streptomycin and 7,5µg/ml puromycin (Sigma Aldrich) to select for shRNA positive cells. Cells were kept under puromycin selection for passaging throughout the study.

RNA isolation and qRT-PCR

RNA was isolated using Qiashredder and RNeasy Kit (Qiagen) and was reverse transcribed using oligo dT primers and RevertAid Reverse Transcriptase (both Fermentas). qRT-PCR was performed using SensiMix SYBR Green (Bioline) in technical triplicates analyzed on Rotor-Gene Q from Qiagen. Results were normalized to the housekeeping gene cyclophilin B (Ppib) (Table S1).

Cytokine measurement

Stimulated cell supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) using mouse or human IL-6 ELISA Ready-SET-Go (eBioscience) and mouse CCL5/RANTES (R&D Systems) according to the manufacturer's instructions.

Il6 transcription measurements

5×10^5 RAW cells were seeded, incubated in serum-free media for 2 hours and stimulated with LPS (100ng/ml) or CpG (5μM) for indicated time points. *Il6* mRNA expression was measured by qRT-PCR (see above).

Confocal microscopy

Stable shRNA expressing RAW cells were seeded on glass coverslips overnight. Cells were washed and incubated with serum free media for 2 hours prior to 16 hours stimulation with LPS (100ng/ml). Coverslips were washed, then fixed, and permeabilized with 4% formalin/0.1% TritonX-114 in PBS. IL-6 was visualized with directly conjugated APC anti-Mouse-IL-6 (MP5-20F3, BD Biosciences), and actin with anti-pan actin (Cytoskeleton, Inc); secondary anti-rabbit AlexFlour594 (Invitrogen). Slides were visualized using an LSM700 (Carl Zeiss) utilizing sequential laser line interrogation into two MPTs. Images were taken at 63× and analyzed with ImageJ (NIH, open source).

Lipid supplementation

All lipids were purchased from Avanti Polar Lipids and solubilized as previously described using Ethanol/Dodecane (Wijesinghe et al., 2009). Briefly, long-chained lipids (starting from a fatty acid chain length of C16) were sonicated for 25 minutes at 40°C and vortexed every 5 minutes. For the lipid supplementation assay lipids (15μM) were added to a 96-well microtiter plate in 50μl serum-free media. Each condition was performed in technical quadruplicates per experiment. 5×10^5 RAW264.7 cells/ml were seeded and incubated for 30 minutes in indicated lipid concentrations prior to stimulation. Cells were stimulated with LPS (100ng/ml) for 8 hours.

Lipidomics

RAW264.7 cells and human fibroblasts were grown to 80% confluence and after washing first with PBS then with serum free medium and then were incubated in serum free medium for 2 hours. The cells were harvested, washed with PBS and counted. Sample preparation was done using a methanol/chloroform extraction protocol with 20µl sample volume of frozen cell pellets according to the manufacturer's instructions (BIOCRATES Life Sciences AG, Innsbruck, Austria). The non-diluted extracts were measured with two acquisition methods, and the diluted extracts were measured with a third acquisition method. Targeted metabolomics analysis was performed using the validated in-house method LIPIDS analyzed on an AB SCiex triple-quadrupole mass spectrometer operating in positive and negative MRM mode (BIOCRATES Life Sciences AG, Innsbruck, Austria). 43 calibrators in 7 levels and 5 internal standards (3 of them were deuterated) were used to measure a panel of glycerophospholipids and sphingolipids. 5 quality controls were measured after 20 samples to improve the quality of the measurement. Data analysis was performed using the MetIDQ software (BIOCRATES Life Sciences AG, Innsbruck, Austria). An isotope-correction tool was included to correct and recalculate the measured signals, to avoid any influence of neighbored MRMs and to ensure the quality of the measurement.

Flow cytometry

For analysis of TLR4 PM levels RAW264.7 cells were stimulated with LPS (100ng/ml) for indicated time points in a 96-well microtiter plate at 37°C. After washing with cold PBS, nonpermeabilized cells were incubated with anti-TLR4-PE antibody (clone MTS510 from BioLegend) for 20 minutes on ice. Cells were then analyzed on FACS Fortessa measuring 10,000 cells per sample.

Statistics and computational analysis

Annotation enrichment analysis

Annotation enrichment analysis was performed on the differentially expressed genes (at $p<0.05$ and an absolute $\log_2$(fold-change) in expression ≥ 1.5) upon TLR4 stimulation of BMDMs as published on systemsimmunology.org, for any of the differentially expressed genes found between 2 and 6 hours post-stimulation. Annotation enrichment analysis was calculated using the webservice DAVID (http://david.abcc.ncifcrf.gov/home.jsp) with default settings. Reported are the top-enriched annotations containing the word "lipid" and selected non-enriched annotations.

ShRNA phenotype calculation per gene

Experiments were filtered based on viability and on the induction of IL-6 release upon TLR stimulation for sh:GFP. ShRNA phenotypes per gene were calculated by either averaging out the ELISA results from all technical replicates from the most recent biological replicate, or from the two or more shRNAs per gene that displayed absolute $\log_2$ fold-changes of above 0.7 over the corresponding sh:GFP with a consistent sign (i.e. consistently increasing or decreasing IL-6 release) within a single experimental repeat and consistent between repeated experiments.

Lipidomics data normalization

The lipidomics results were normalized based on the sum of concentrations for all lipid species measured in a single biological replicate. Values were next averaged over the three biological replicates, and $\log_2$ transformed against the corresponding average concentrations measured in sh:GFP.

Hierarchical clustering and Hierarchical Interaction Score

Hierarchical clustering was performed using Matlab on non-normalized data using correlation as distance measure and average linkage. Hierarchical interaction scores (HIS) were calculated as described in (Liberali et al., 2014; Snijder et al., 2013). Specifically, interaction scores were calculated between genes from z-score normalized $\log_2$(FC) transformed lipidomics results, thresholding between

z-scores from 1 to 3 on both the positive and negative end of the data distribution (i.e. default settings), Edges scored with HIS < 0.3 were discarded, and all remaining edges are shown.

Lipid coregulation network analysis

Lipid coregulation (as visualized in the circular correlation network) was defined as correlation values of 0.7 or higher. Various natural network layout algorithms (as implemented in Cytoscape) were tested to confirm that the circularity shown was not an artefact from the selected network layout optimization algorithm. The threshold of 0.7 was tested to be high enough to be sufficiently robust to leaving out the single assay that most contributed to any one correlation value, and therefore likely not solely determined by the phenotype of any single cell line.

Network clustering significance

The significance of clustering of various features per node (i.e. lipid) was calculated by comparing the absolute difference of the given feature between a node and its nearest neighbor as defined by the network, averaged over all nodes, with the distribution of over 10,000 repeats of the same calculation using randomly shuffled feature values. If the actual nearest neighbor distance was lower than the lowest value observed in the 10,000 randomized repeats, the significance of this difference was calculated by two-tailed t-test between the actual observation and the >10,000 repeats. This was confirmed to approximate true values, and a reasonable approximation as all randomized distributions were highly normally distributed.

Lipid subcellular membrane fraction enrichment score

The lipid subcellular membrane fraction enrichment scores were calculated as the z-score over the lipid concentrations measured for any one lipid species over all the different fractions analyzed by the lipidmaps.org consortium.

Lipid function prediction

Functional predictions or associations for lipids were performed based on Pearson's linear correlation coefficients between the $\log_2$(FC) readouts of the TLR4-related functional assays (i.e. IL-6 release, *Il6* transcription, TLR4 surface levels) and the $\log_2$(FC) in lipid levels, over the 9 shRNA cell lines.

General statistics

P-values were calculated with two-tailed t-tests, unless otherwise indicated. Correlation values given are Pearson's linear correlation coefficients, unless otherwise indicated. The significance of the overlap in lipid-lipid coregulation between the RAW264.7 dataset and the fibroblast dataset were calculated by the hypergeometric distribution.

Supplemental References

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