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Supplementary Information for

Triglyceride breakdown from lipid droplets regulates the inflammatory response in macrophages

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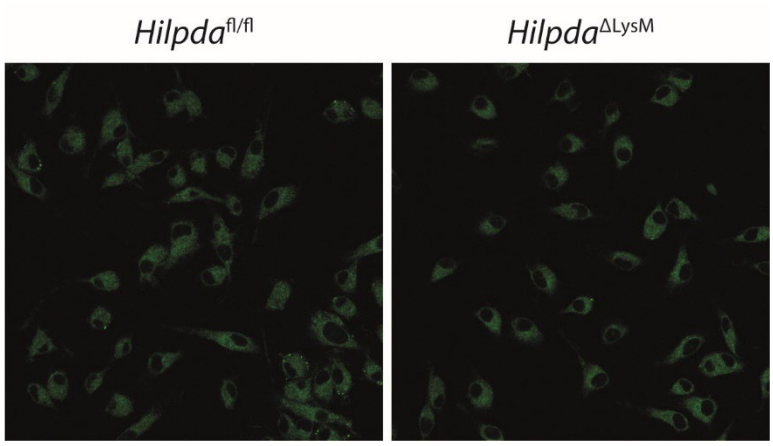
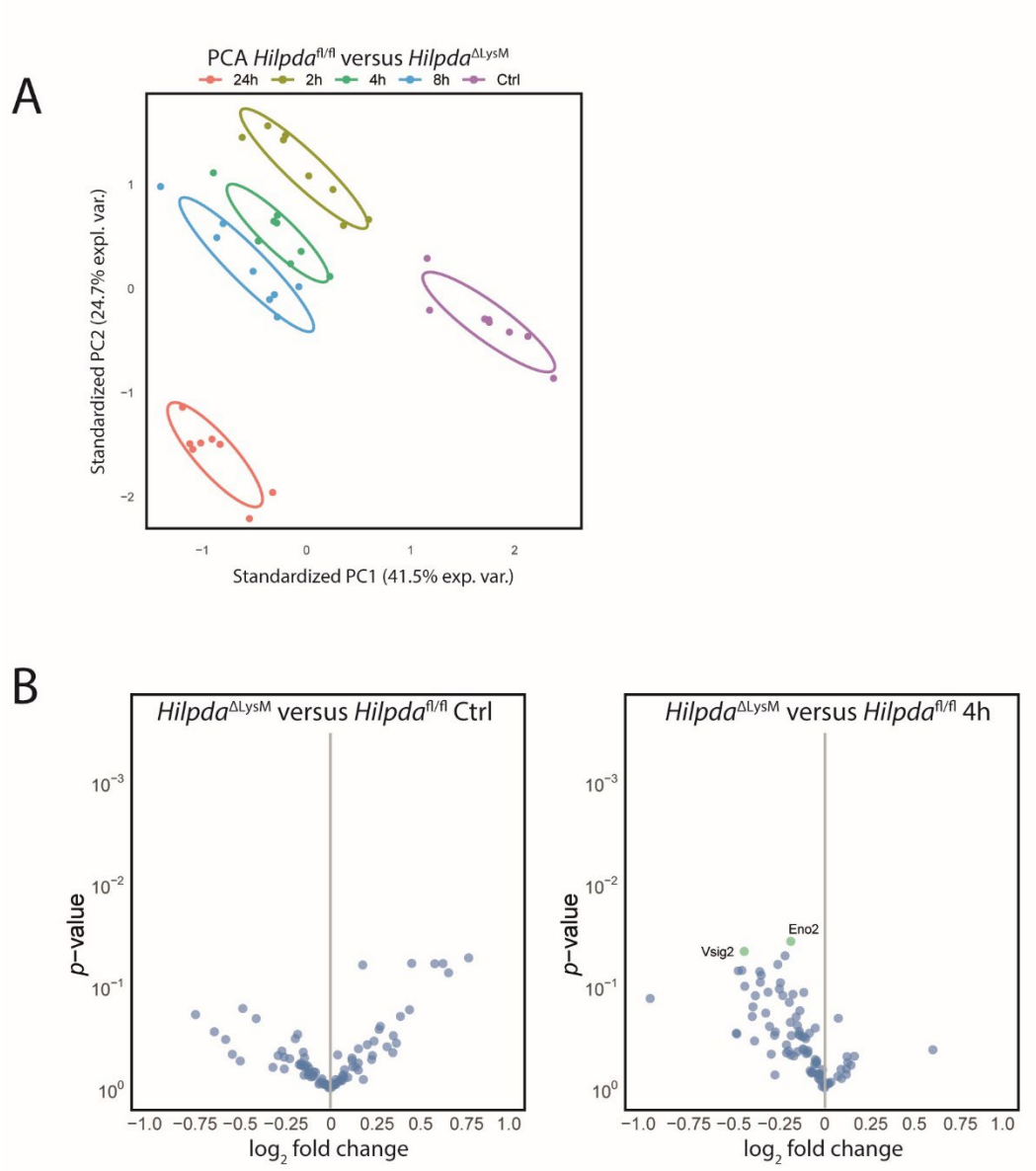
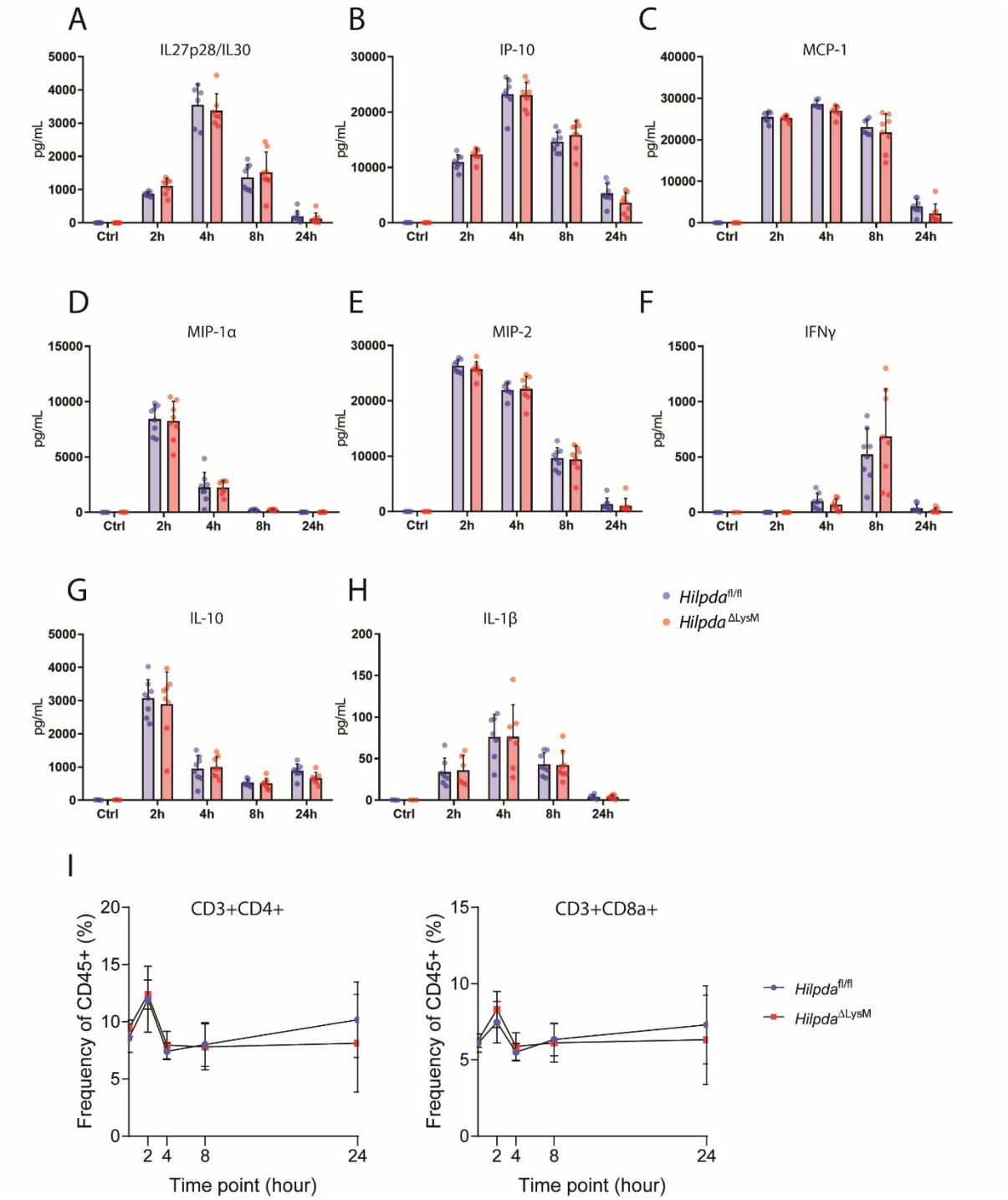
Figures S1 to S5



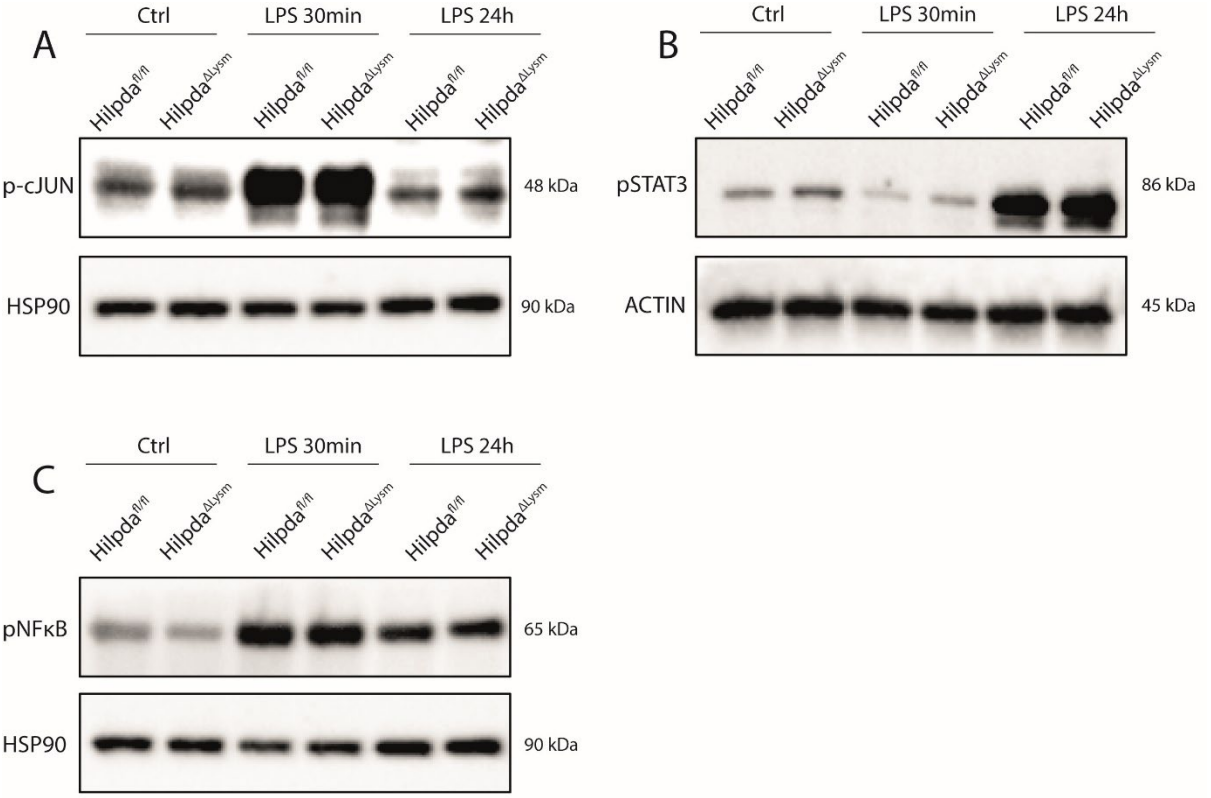
Supplemental Figure 1, corresponds to Figure 2. BODIPY staining of *Hilpda*$^{fl/fl}$ (A) and *Hilpda*$^{\Delta LysM}$ (B) BMDMs treated with vehicle for 24h.



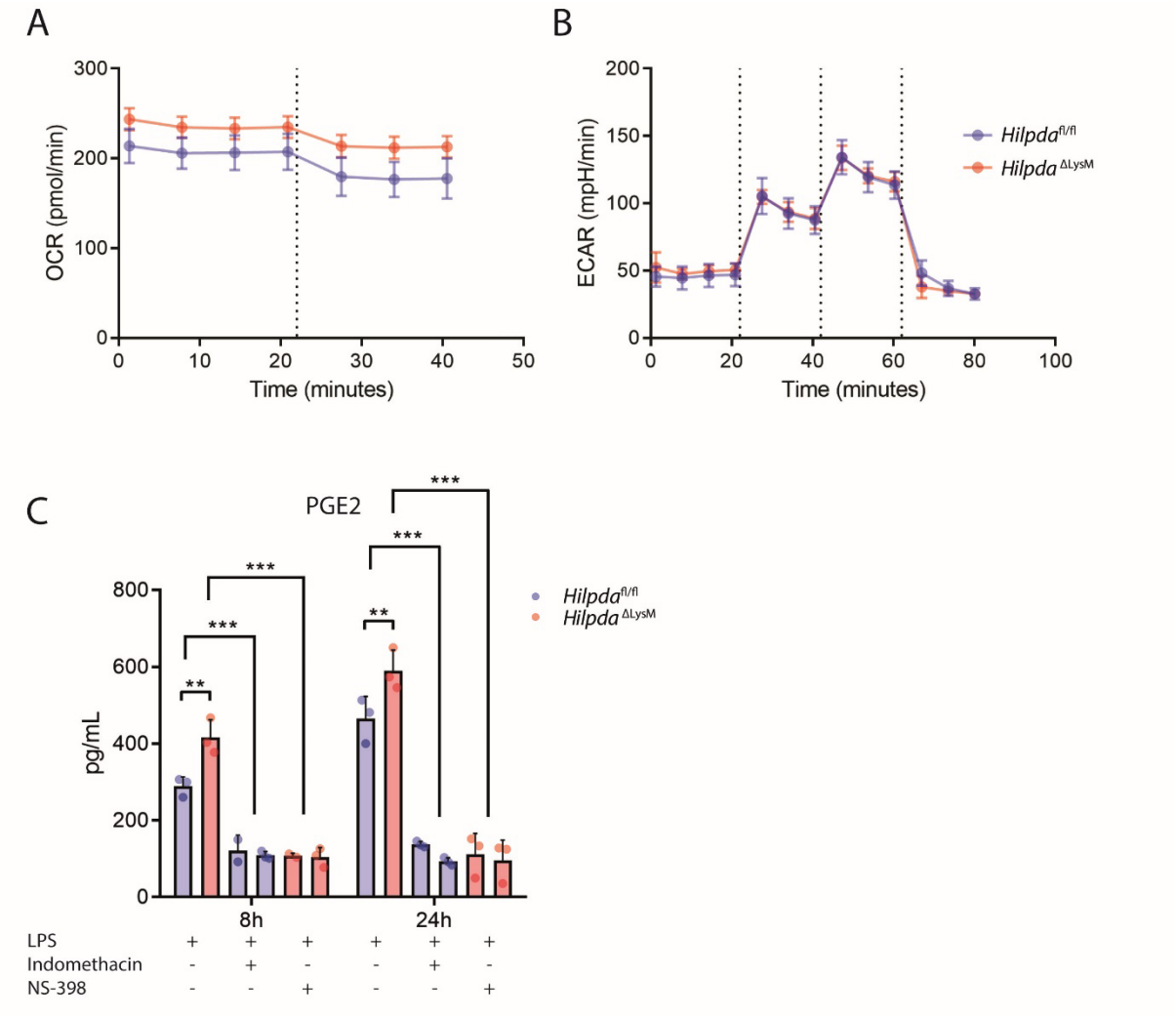
Supplemental Figure 2, corresponds to Figure 3. (A) PCA of targeted plasma proteomic profiles (Olink) in *Hilpda*$^{\Delta LysM}$ and *Hilpda*$^{fl/fl}$ mice (grouped per time point) 2, 4, 8 or 24h after intraperitoneal injection of LPS or 24h after intraperitoneal injection of saline (Ctrl). (B) Relative fold change and uncorrected *p*-values in plasma proteomic profiles in *Hilpda*$^{\Delta LysM}$ and *Hilpda*$^{fl/fl}$ mice 4h after intraperitoneal injection of LPS, or 24h after intraperitoneal injection of saline (Ctrl). Ctrl: control; PCA: principal component analysis.



Supplemental Figure 3, corresponds to Figure 4. (A - H) Plasma concentration of IL27p28/IL30, IP-10, MCP-1, MIP-1α, MIP-2, IFNγ, IL-10 and IL-1β in Hilpda$^{\Delta LysM}$ and Hilpda$^{fl/fl}$ mice 2, 4, 8 or 24h after intraperitoneal injection of LPS or 24h after intraperitoneal injection of saline (Ctrl). (I) Relative circulating T cell subpopulations in *Hilpda*$^{\Delta LysM}$ and *Hilpda*$^{fl/fl}$ mice 2, 4, 8 or 24h after intraperitoneal injection of LPS or 24h after intraperitoneal injection of saline (baseline). Bar graphs are represented as mean ±SD. Ctrl: control.



Supplemental Figure 4. Protein expression of (A) p-cJUN, (B) p-STAT3 and (C) p-NFκB in *Hilpda*$^{\Delta LysM}$ and *Hilpda*$^{fl/fl}$ BMDMs after treatment with vehicle (Ctrl) or LPS for 30 min or 24h. HSP90 and ACTIN are used as loading controls. Ctrl: control; LPS: lipopolysaccharide.



Supplemental Figure 5. (A) Oxygen consumption rate of *Hilpda*$^{\Delta LysM}$ and *Hilpda*$^{fl/fl}$ BMDMs after treatment with LPS for 24h, measured by extracellular flux analysis. Injection of etomoxir is indicated with dotted line. (B) Extracellular acidification rate of *Hilpda*$^{\Delta LysM}$ and *Hilpda*$^{fl/fl}$ BMDMs after treatment with LPS for 24h, measured by extracellular flux analysis. Injection of glucose, oligomycin and 2-deoxyglucose are indicated with dotted lines. (C) Concentration of PGE2 derived from *Hilpda*$^{\Delta LysM}$ and *Hilpda*$^{fl/fl}$ BMDMs after treatment with LPS and indomethacin or NS-398 for 8 or 24h. Line and bar graphs are represented as mean ±SD. OCR: oxygen consumption rate; ECAR: extracellular acidification rate; LPS: lipopolysaccharide; PGE2: prostaglandin-E2. **p<0.01, ***p<0.001

Primary Cell Isolation

Peritoneal macrophages were harvested by flushing the peritoneal cavity with ice-cold phosphate-buffered saline (PBS). The macrophage fractions were pooled from two mice from the same group, and purified by magnetic selection using an anti–F4/80-FIT C antibody (130-117-509; RRID: AB_2727970; Miltenyi Biotec), anti-FITC MicroBeads (130-048-701; RRID: AB_244371; Miltenyi Biotec), and MS columns (Miltenyi Biotec) on the OctoMACS Cell Separator System (Miltenyi Biotec). Peritoneal macrophages were plated in 96-well plates for collection of supernatant or in 8-well glass-bottom µ-slides (Ibidi) for confocal imaging, and cultured in Roswell Park Memorial Institute (RPMI)-1630 medium (Lonza) supplemented with 10% fetal calf serum (FCS; BioWest) and 1% penicillin/streptomycin (P/S; Corning) at 37 °C and 5% CO2 for 24 h. The supernatant was collected and stored at −80 °C; 96-well plates were stored at −20 °C. Macrophages in glass-bottom slides were fixed in 3.7% paraformaldehyde, washed with PBS, and stored at 4 °C before staining.

After the mice were killed, spleens were stored on ice in RPMI-1630 supplemented with 10% FCS and 1% P/S. Spleens were strained through 100-µm cell strainers, and RBCs were lysed with RBC lysis buffer (eBioscience, Thermo Fisher Scientific). Splenic macrophages were isolated by magnetic selection using an anti–F4/80-FIT C antibody (130-117-509; RRID: AB_2727970; Miltenyi Biotec), anti-FITC MicroBeads (130-048-701; RRID: AB_244371), and MS columns (Miltenyi Biotec) with the OctoMACS Cell Separator System (Miltenyi Biotec). Splenic macrophages were centrifuged, and cell pellets were directly dissolved in TRIzol reagent (Invitrogen, Thermo Fisher Scientific) and stored at −80 °C.

For the isolation of BMDMs for in vitro experiments, 8- to 12-wk-old wild-type, *Hilpda*$^{\Delta LysM}$, or *Hilpda*$^{fl/fl}$ mice were killed by cervical dislocation and hind legs were isolated at the hip joint. Bone marrow was flushed from femur and tibia and differentiated in Dulbecco's modified Eagle's medium (Corning) supplemented with 10% FCS, 1% P/S, and 15% L929 cell line–conditioned medium. After differentiation for 7 d, cells were scraped and plated as appropriate.

Functional Assays

BMDMs were treated with LPS (100 ng/mL) for 24 h to induce LD accumulation. Apoptosis was measured in BMDMs from *Hilpda*$^{\Delta LysM}$ and *Hilpda*$^{fl/fl}$ mice by incubating with 2.5 ng/µL fluorescein isothiocyanate (FITC)–conjugated Annexin-V-FP488 AQ29, kindly provided by C. Reutelingsperger, University of Maastricht, Maastricht, The Netherlands (35), for 15 min in Annexin binding buffer, adding 300 nM staurosporine (from *Streptomyces* sp.; S4400; Sigma-Aldrich) to positive-control wells. Staining was quenched with 200 mM potassium iodide. Efferocytosis was measured by staining Jurkat E6.1 cells (88042803; Sigma-Aldrich) with calcein (AM; C1430; Invitrogen, Thermo Fisher Scientific) and rendering them apoptotic by treatment with staurosporine (from *Streptomyces* sp.; S4400; Sigma-Aldrich) at a concentration of 5 µM for 1 h. Jurkat cells were coincubated for 1.5 h with BMDMs from *Hilpda*$^{\Delta LysM}$ and *Hilpda*$^{fl/fl}$ mice (~3 Jurkat cells per BMDM). Noninternalized Jurkat cells were detached and BMDMs were stained with CD11b-Alexa Fluor 647 (101218; BioLegend). Phagocytosis was assessed by incubating cells with pHrodo Red Zymosan Bioparticles (P35364; Life Technologies, Thermo Fisher Scientific) for 1 h, while cytochalasin D (C2618; Sigma-Aldrich) was added to negative control wells to inhibit phagocytosis. For all assays, nuclei of BMDMs were stained with Hoechst 33342 (B2261; Sigma-Aldrich). All the above-described functional assays were processed on the BD Pathway 855 High Content Analyzer (BD Biosciences) using the 10-fold objective, making 9 images per well (3 × 3). Data were analyzed with AttoVision software (BD Biosciences), FACSDiva software (BD Biosciences), and CellProfiler software (43). Briefly, background signals were subtracted, flat-field correction was performed, cells were segmented on each image based on the nuclei, and the intensity of the stainings was recorded.

Data were analyzed in FACSDiva software, calculating the mean fluorescence intensity and percentage of positive cells. For the efferocytosis assay, CellProfiler software was used to calculate the average number of internalized Jurkat cells per BMDM.