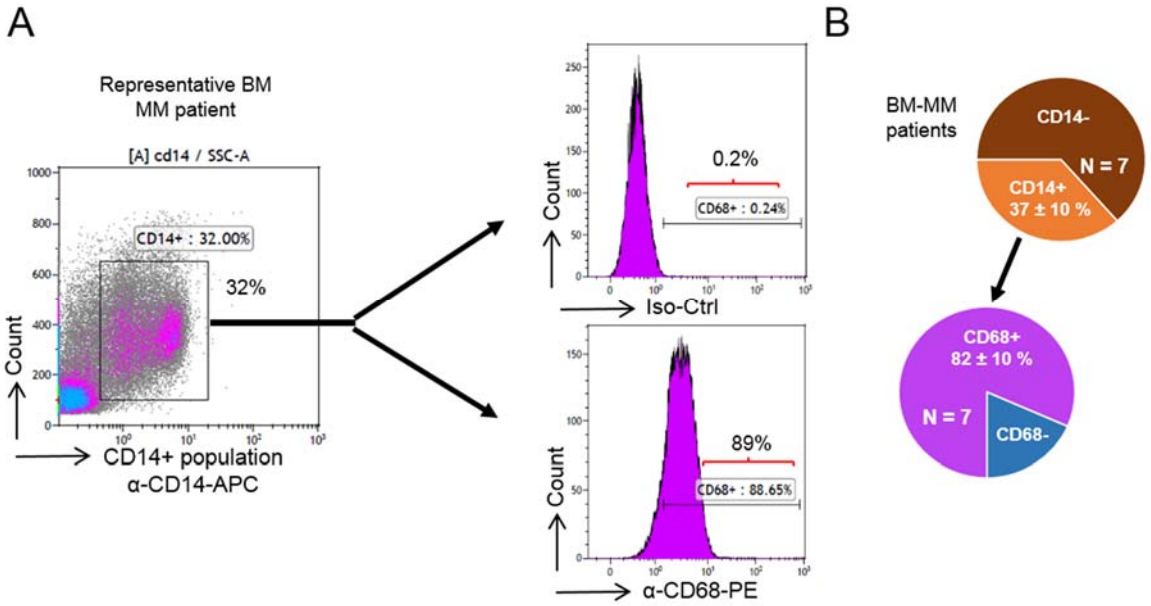
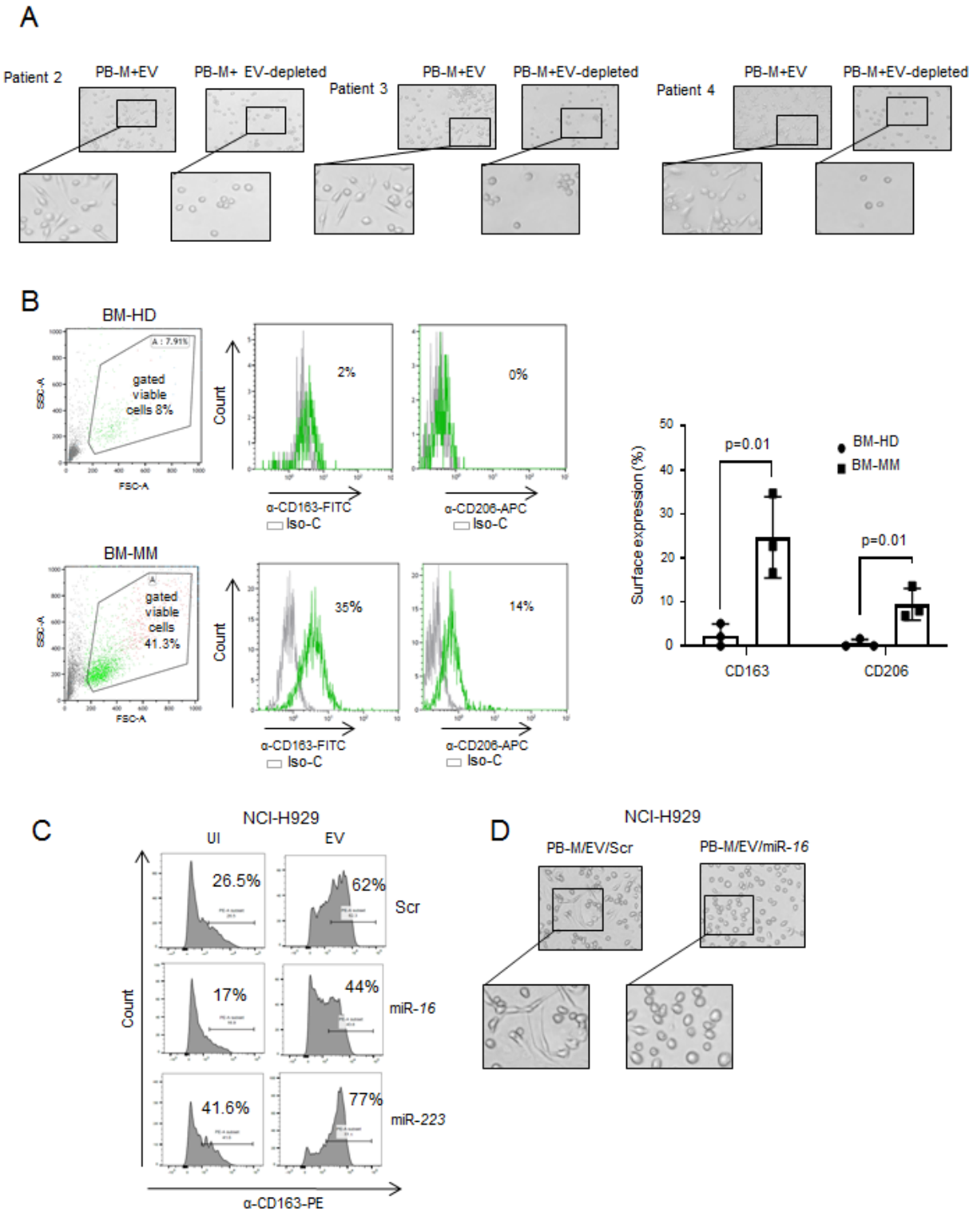
Supplemental Figures



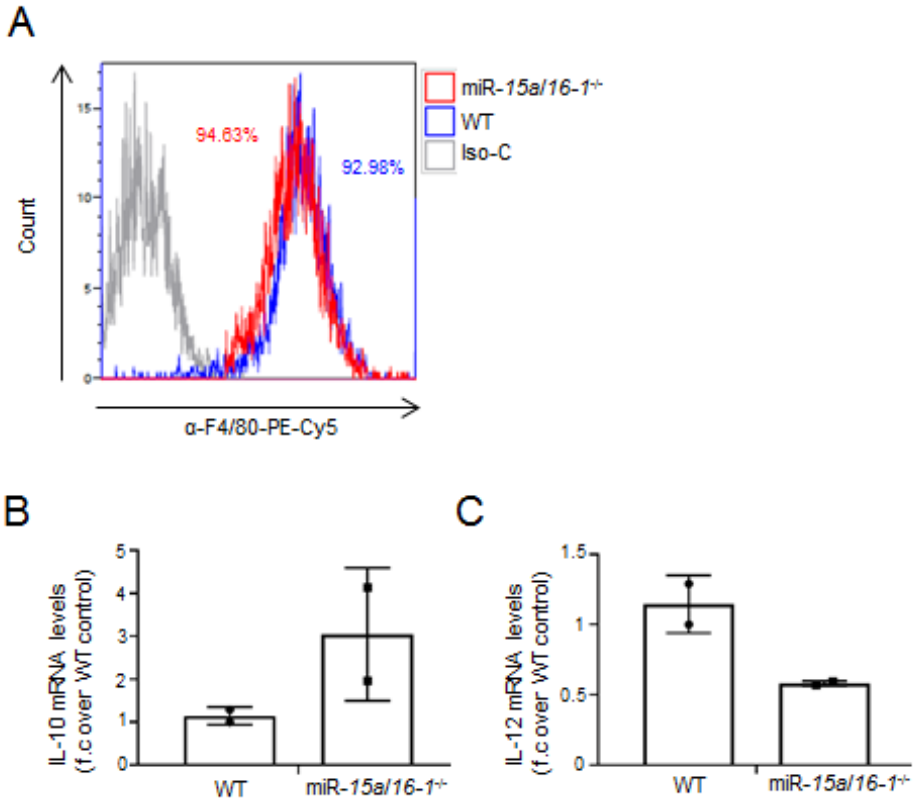
Supplemental Figure 1

(A) On the left, representative flowsight cytometric analysis showing percent of CD14+ cells in total BM cellularity obtained from n=7 MM patients. On the right, representative flow cytometry histograms showing the percent of the MΦ marker CD68 expressed in the CD14+ cell population (bottom right panel) gated using isotype control (Iso-Ctrl) (top right panel). (B) Pie chart showing the percent of CD14+ population ± SD in BM of MM patients as well as the percent of CD68+ cells ± SD present in the CD14+ fractions (n=7 patients).


A
Patient 2
PB-M+EV
PB-M+ EV-depleted
Patient 3
PB-M+EV
PB-M+EV-depleted
Patient 4
PB-M+EV
PB-M+EV-depleted
B
BM-HD
gated viable cells 8%
SSC-A
FSC-A
Count
2%
0%
α-CD163-FITC
Iso-C
α-CD206-APC
Iso-C
BM-MM
gated viable cells 41.3%
35%
14%
p=0.01
p=0.01
BM-HD
BM-MM
Surface expression (%)
CD163
CD206
C
NCI-H929
UI
EV
26.5%
62%
Scr
17%
44%
miR-16
41.6%
77%
miR-223
Count
α-CD163-PE
D
NCI-H929
PB-M/EV/Scr
PB-M/EV/miR-16

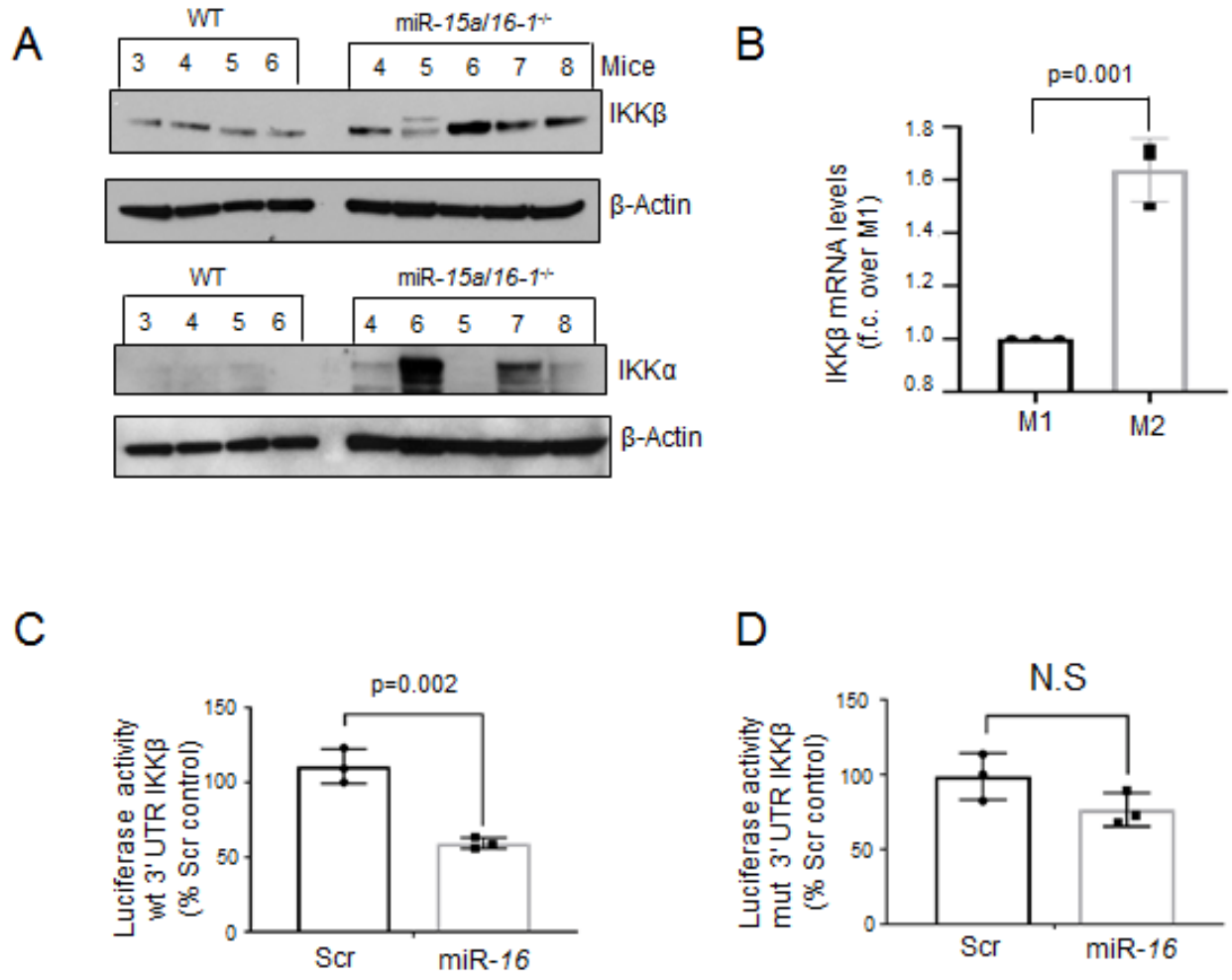
Supplemental Figure 2

(A) Representative images captured by light microscopy showing differentiation of PB-M obtained from an HD incubated with EV isolated from the BM-acellular fraction (BM-ac) of three different MM patients (patient 2, patient 3, patient 4) (PB-M+EV; left panel) or EV-depleted BM-ac from the same patients (PB-M+EV-depleted; right panel). (B) Representative flowsights showing percent of living PB-M when treated with the conditional media of cultured total BM cellular population isolated from cancer free donor (BM-HD left upper) or MM patient (BM-MM lower panels). Representative histograms overlaid with the respective isotype control in gray (Iso-C) showing comparable CD163 and CD206 (green) percent surface expression. Gating strategy was set using a mix of IgG anti-FITC and IgG anti-APC antibody isotype controls. (B-right panel). Bar dot plots showing average CD163 and CD206 percent surface expression on differentiated PB-M treated with the conditional media of BM-HD or BM-MM (n=3/group). Values represent the mean ± SD; the reported p values were calculated using unpaired t-test (tails = 2). (C) Flow cytometric analysis showing percent surface expression of CD163 on PB-M differentiated with EV isolated from the conditioned media of NCI-H929 MM cells and concomitantly incubated with either ds-miR-16 (middle right panel), ds-miR-223 (lower right panel), or Scr control (upper right panel). CD163 percent surface expression on undifferentiated cells (UI) incubated with the microRNAs cited above are also shown (left panels). Gating strategy was set using IgG anti-PE antibody isotype control. (D) Representative images captured by light microscopy showing impairment of PB-M differentiation to M2-MΦ in the presence of ds-miR-16. PB-M were incubated with the EV isolated from NCI-H929 cells and treated with ds-miR-16 (right panel) or Scr control (left panel) for 7 days.



Supplemental Figure 3

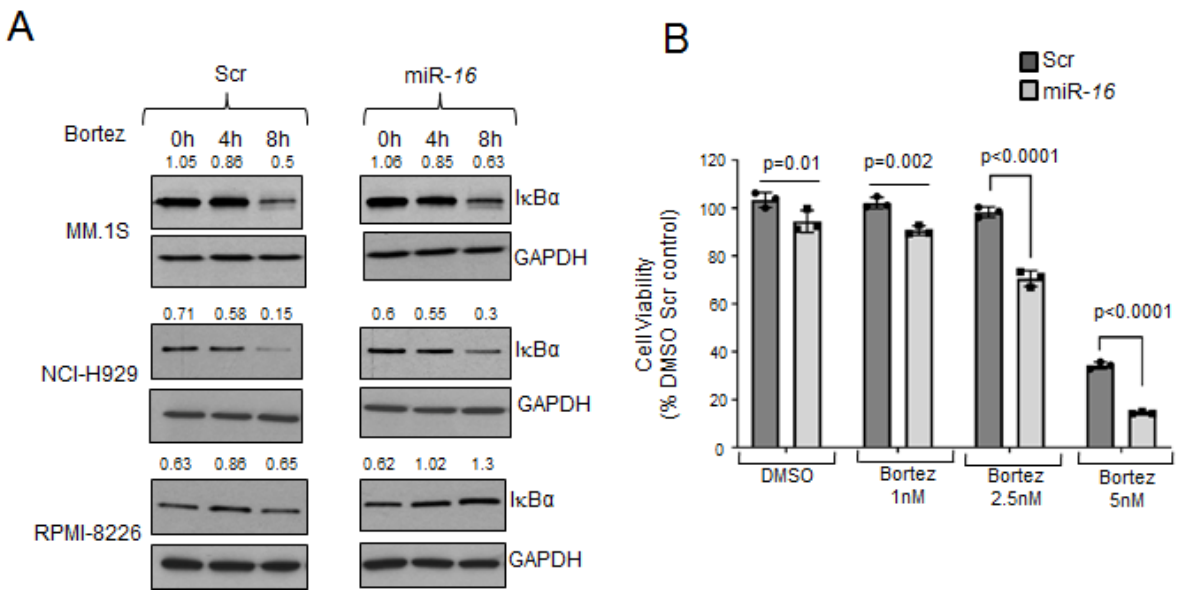
(A) Representative overlaid flowsight cytometric analysis showing percent of M0-MΦ F4/80 positive cells isolated from the spleen of WT and miR-15a/16-1-/- mice (n=4/group). Gating strategy was set using an IgG anti-PE-Cy5 antibody isotype control. (B-C) Quantitative RT-PCR analysis showing mRNA expression of IL-10 (B) and IL-12 (C) in differentiated MΦ isolated from WT or miR-15a/16-1-/- B6 mice (n=4 mice/group); the RNA from each two mice/group were pooled together to ensure the quality of the results, and a triplicate PCR reading was done for each pool. Error bars represent variance.



Supplemental Figure 4

(A) Western blot analysis showing protein levels of IKKβ and IKKα in monocyte/MΦ CD11b fractions isolated from additional spleens obtained from WT or miR-15a/16-1-/- mice (n=4 WT; n=5 miR-15a/16-1-/- mice). β-Actin is used as loading control. (B) Quantitative RT-PCR analysis showing mRNA expression of IKKβ in PB-M isolated from MM patients differentiated to M1 or M2-MΦ with GM-CSF (granulocyte macrophage colony stimulating factor) or M-CSF (macrophage colony stimulating factor), respectively. (n=3 patients). (C-D) Luciferase reporter assay showing IKKβ 3'UTR transcriptional activity in HeLa cells transfected with pGL4.11 luciferase vector containing miR-*16-5*p WT (C) or mutated (D) 3'UTR IKKβ binding site. HeLa cells were transfected

with the corresponding luciferase vectors for 12hrs followed by a second transfection with ds-miR-16 or Scr control for an additional 12hrs. Transfection efficiency was controlled by co-transfection with TK promoter-Renilla vector. Data are presented as percent of Scr control. For each reported experiment values represent the mean ± SD; the reported p values were calculated using unpaired t-test (tails = 2). Each experiment was performed in triplicate. N.S.= Non Significant.



Supplemental Figure 5

(A) Western blot analysis showing protein levels of IkBα in three MM cell lines (MM.1S, NCI-H929 and RPMI-8226) transfected with miR-16 or Scr for 24h and treated with 20 nM bortezomib in a time-dependent manner as indicated in the figure (0 hours, 4 hours, 8 hours). GAPDH was used as loading control. Numbers indicated above each band represent the fold change in band intensity relative to GAPDH determined using densitometric analysis. (B) MTS assay showing viability of MM.1S cells upon transfection with miR-16 or Scr sequences treated with different doses of bortezomib (1, 2.5, and 5 nM) or DMSO veh control for 48hrs. Data are presented as percent of DMSO Scr control. Values represent the mean ± SD; the reported p values were calculated using one-way ordinary ANOVA multi-comparisons test.

Supplemental Methods

Flow cytometry

For CD163 expression analysis in primary samples and cell lines, cells were washed with PBS and stained for 30 minutes using CD163-PE mouse anti-human antibody (Cat# 556018, BD Biosciences). Cells were washed and immediately analyzed on LSRII (Becton Dickinson). Analysis was conducted using FlowJo Software (Treestar, Ashland, OR).In Figure 4 and Supplemental Figure 2B, CD163-FITC mouse anti-human antibody (Cat#563697, BD Biosciences) and CD206-APC mouse anti-human antibody (Cat#550889, BD Biosciences) were used. Analysis was conducted using Kaluza Software (Beckman Coulter). Percentages of CD163 and CD206-positive cells were determined for each treatment condition.

MM.1S cells transfected with miR-16-5p mimic or scramble control and treated on day 2 with 2.5 nM bortezomib for 24hrs were analyzed for apoptosis by Annexin-V-APC conjugated/propidium iodide (PI) staining assay according to the manufacturer's protocol (Cat.#A35110, ThermoFisher Scientific).Analysis was conducted using FlowJo Software (Treestar, Ashland, OR). Percent of Annexin-V positive cells were determined for each treatment condition.

For spleen MΦ isolated from wild type C57BL/6 (WT) mice or miR-*15a*/*16-1* knockout (KO) mice, cells were washed with PBS and stained for 30 minutes using the following antibodies: F4/80-PE/Cy5rat anti-mouse antibody (Cat# 123112, Biolegend); Dectin-1-APC rat anti-mouse antibody (Cat#144306, Biolegend); CD206-Alexa Fluor 488 rat anti-mouse antibody (Cat# 141710, Biolegend). Cells were washed and immediately analyzed on LSRII (Becton Dickinson). Analysis was conducted using Kaluza Software (Beckman Coulter). F4/80 antibody was used for determination of MΦ purity. Percent of Dectin-1 and CD206-positive cells expression were determined.

For human specimens, the isotype controls used are:

1- PE-Mouse anti-human IgG isotype control antibody (BD Biosciences, cat# 559320)
2- FITC-Mouse IgG isotype control antibody (Invitrogen, cat# MG101)
3- APC-Mouse anti-human IgG isotype control antibody (BD Biosciences, cat# 561323)

For mouse specimens, the isotype controls used are:

1- Alexa Fluor 488-Armenian Hamster IgG isotype control antibody (Biolegend, cat# 400923)
2- PE-Cy5-Armenian Hamster IgG isotype control antibody (Biolegend, cat# 400909)
3- APC-Armenian Hamster IgG isotype control antibody (Biolegend cat# 400911)

Viability assay

Cell viability was assessed using the MTS cell proliferation assay (Promega) according to the manufacturer's protocol.

Luciferase assay

HS-5 or HeLa cells were transfected with 500 ng 3xNFkB-pGL3wt, 3xNFkB-pGL3 or 3'UTR IKKβ-pGL4.11-based luciferase vectors (see DNA constructs section in main text for more details) and 50 ng of Renilla luciferase expression construct (pRL-TK; Promega), using Lipofectamine 3000 (Invitrogen). At the time of harvest (see figure legends) cells were lysed and tested by Dual Luciferase Assay (Promega), according to the manufacturer's instructions. U266 cells were transfected with 1.8 μg of 3'UTR-IKKβ-pGL4.11-based luciferase vector and 200 ng of pRL-TK. At the time of harvest (see figure legends), cells were assayed for luciferase activity as described above.

Immunoblotting

Cells were harvested by centrifugation, washed with PBS, and lysed using buffer composed of 50 mM Tris (pH7.5), 150 mM NaCl, 10% glycerol, 1.0% NP-40, and 0.1% SDS and supplemented with protease and phosphatase inhibitors. Protein concentrations were estimated by BCA assay, and equivalent quantities of the lysates were resolved on 4–12% Bis-Tris Plus Gels (Cat# NW04122, Invitrogen). Proteins were transferred to PVDF membranes and stained for IKKα (Cat#sc-7606, Santa Cruz Biotechnology), IKKβ (Cat# D30C6, Cell Signaling Technology), IKBα (Cat#sc-371 Santa Cruz Biotechnology), glyceraldehyde 3-phosphatedehydrogenase (GAPDH, Cat#sc-47724, Santa Cruz Biotechnology), or β-Actin (Cat#sc-47778, Santa Cruz Biotechnology) followed by anti-mouse or anti-rabbit IgG-HRP (GE Healthcare). Signals were developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific). For IKKα detection of proteins originated from mice tissues, IKKα primary antibody (Cat#2682, Cell Signaling Technology) was used.

Nuclear protein extraction was done using a nuclear extraction kit (Cat# ab113474, abcam Biotechnology) according to the manufacturer's instructions. Additionally, after separation of the nuclear and cytoplasmic fractions, the nuclear fraction was washed 8 times before lysis to minimize cross-contamination between the fractions. Protein concentration measurement and immunoblotting were performed as described above. The antibodies used were mouse anti-human p65 (Cat# 6956 Cell signaling Technology) and rabbit anti-human HDAC1 (cat# D5C6U, Cell Signaling Technology).

Cytokine array

The cytokine array was done using the Bio-Plex Pro Human Cytokine assay kit (Cat#15-Q07781V2, BioRad Technology), according to the manufacturer's instructions.

Single cell flow cytometry

MM.1S cells were transfected with miR-16 mimic or scramble control (see Cell transfection section in main text) then stimulated with human recombinant TNF-α (10ng/ml) for 45 minutes. Cells were assessed for p65 nuclear translocation following published protocol(1). Briefly, cells were then washed and fixed with 4% formaldehyde for 10 min at room temperature, then permeabilized with 0.1% Triton X-100 in PBS with 3% FBS for 40 minutes. Fixed permeabilized cells were then stained with primary rabbit anti-human p65 antibody (Cat# 8242, Cell Signaling Technology) for 30 minutes, washed, and then stained with secondary antibody goat anti-rabbit Alexa-Fluor 647 conjugate (Cat# A-21244, Invitrogen) for 30 minutes. Cells were then washed and stained with DAPI. Acquisition speed was set up to low speed and at the highest resolution, an automated condition provided in Flowsight. Cells were acquired on the basis of area and aspect ratio. Debris and doublets were gated out from the analysis. Channel 1 for Bright field (420-480 nm), channel 11 for Alexa Fluor 647 (660-740 nm), and channel 7 for DAPI nuclear stain (420-505 nm) were used. About 10,000 events of single cells per sample were acquired. Additional single stain labeled samples were prepared, which served as a positive control for single staining of the individual secondary antibodies. Data were analyzed in IDEAS software after compensation of single color control samples using a compensation matrix.

Phagocytosis assay

Cells were plated at a concentration of $1x10^6$ cells/ml in culture medium. A latex beads-IgG-FITC complex (Cat# 400291, Caymanchem) were added to the medium to a final dilution of 1:100. Cells were incubated at 37°C for one hour. Cells were then washed 6 times with PBS, then incubated with trypan blue quenching solution for 2 min, followed by another wash with PBS. Cells were then resuspended in 500 µl PBS 1% formalin and immediately analyzed by flow cytometry on LSRII (Becton Dickinson). Analysis was conducted using FlowJo Software (Treestar, Ashland, OR). Percent of GFP positive cells was detected for each treatment condition as indicated in the

figure legend. For visualization by fluorescence microscopy, 100 µl of cells were plated in 96 well plates, and pictures of GFP-positive cells were taken.

Reference

1. Terrazas C, Oghumu S, Varikuti S, Martinez-Saucedo D, Beverley SM, and Satoskar AR. Uncovering Leishmania-macrophage interplay using imaging flow cytometry. *J Immunol Methods.* 2015;423:93-8.