Supplemental Information

Necroptosis Suppresses Inflammation Via Termination of TNF- or LPS-Induced Cytokine and Chemokine Production

Conor J. Kearney, Sean P. Cullen, Graham A. Tynan, Conor M. Henry, Danielle Clancy, Ed C. Lavelle, and Seamus J. Martin.

Supplemental Information Inventory

1. **Figure S1.** (related to figure 1). MEFs produce TNF-induced cytokines and undergo RIPK3-dependant necroptosis. Also shows that zVAD accelerates TNF-induced necroptosis in L929 cells, using PI staining as a marker of necrosis.
2. **Figure S2** (related to Figure 2). Shows that necroptosis suppresses TNF-induced cytokine production when cells are sensitized using an IAP antagonist. Furtherfore, zVAD does not affect TNF-induced cytokines in the absence of necroptosis (HT-29 cells).
3. **Figure S3** (related to Figure 3). Shows that zVAD does not affect TNF-induced cytokines, in the absence of necroptosis.
4. **Figure S4** (related to Figure 4). Necrostatin-1 inhibits TNF-induced cytokines in MEFs, in the absence of cell death.
5. **Figure S5** (related to Figure 5). Contains the full cell death data set from figure 5. Also shows that zVAD does not affect LPS-induced cytokines, in the absence of necroptosis (THP-1 cells).

Supplemental Data

Figure S1 (Related to Figure 1)

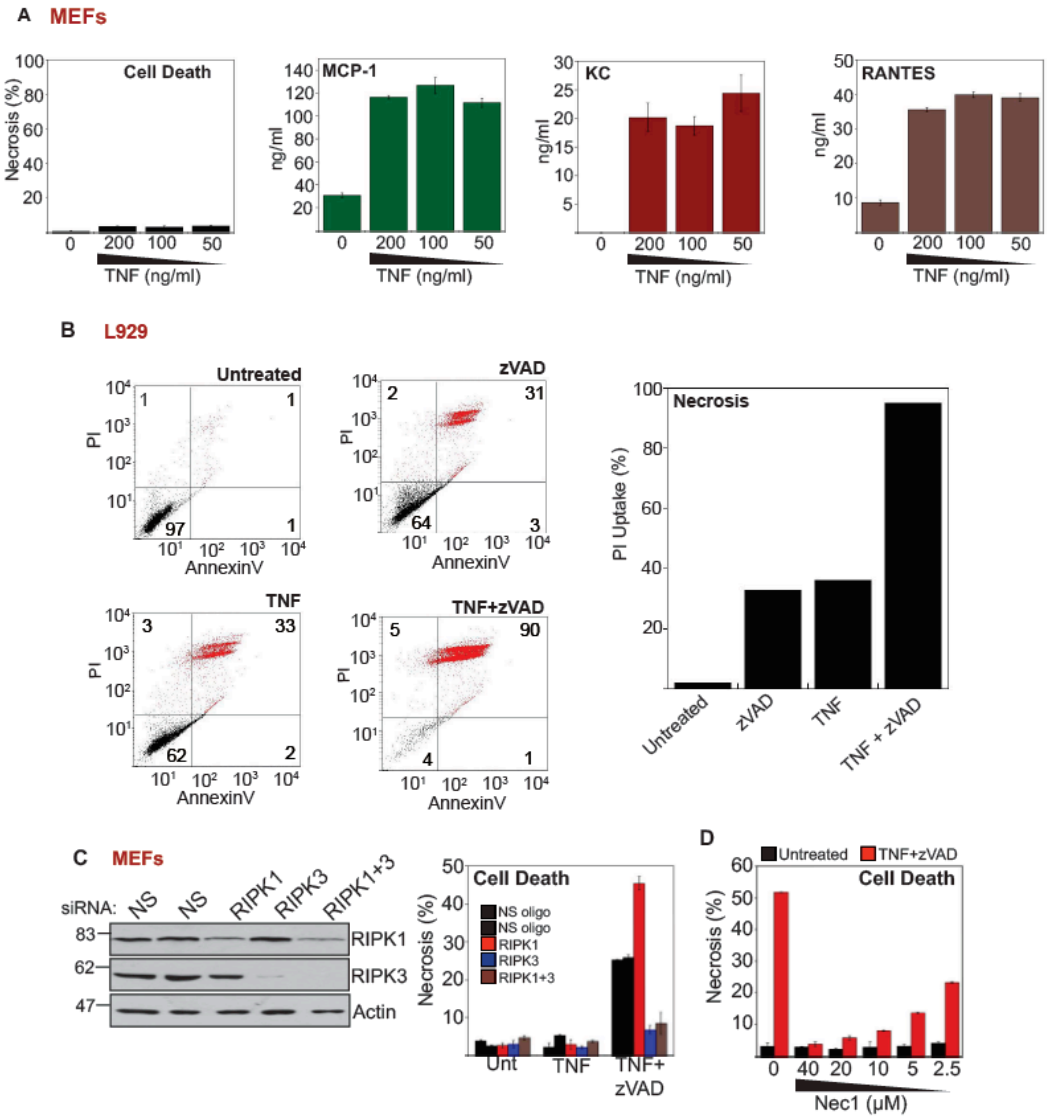

Figure S1. TNF induces cytokine production and necroptosis.
(A) MEFs were treated with the indicated concentrations of TNF. After 24 h, cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA.
(B) L929 cells were pretreated with zVAD (10 μM) for 1 h, followed by addition of TNF (10 ng/ml). After 24 h, cell death was analysed by AnnexinV / Propidium Iodide staining.
(C) MEFs were electroporated with either non-silencing siRNA or siRNA directed against RIPK1, RIPK3 or RIPK1 and RIPK3, as indicated. Fourty eight hours later, cells were pretreated in the presence or absence of zVAD (10 μM) for 1 h, followed by addition of TNF (10 ng/ml). After 5 h, cell death was scored by morphological assessment and cell lysates were analysed by immunoblotting for levels of endogenous proteins.
(D) MEFs were pretreated for 1 h with the indicated concentrations of necrostatin-1 and zVAD (10 μM), followed by addition of TNF (200 ng/ml). After 24 h, cell death was scored by morphology. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

Supplemental Data

Figure S2 (Related to Figure 2)

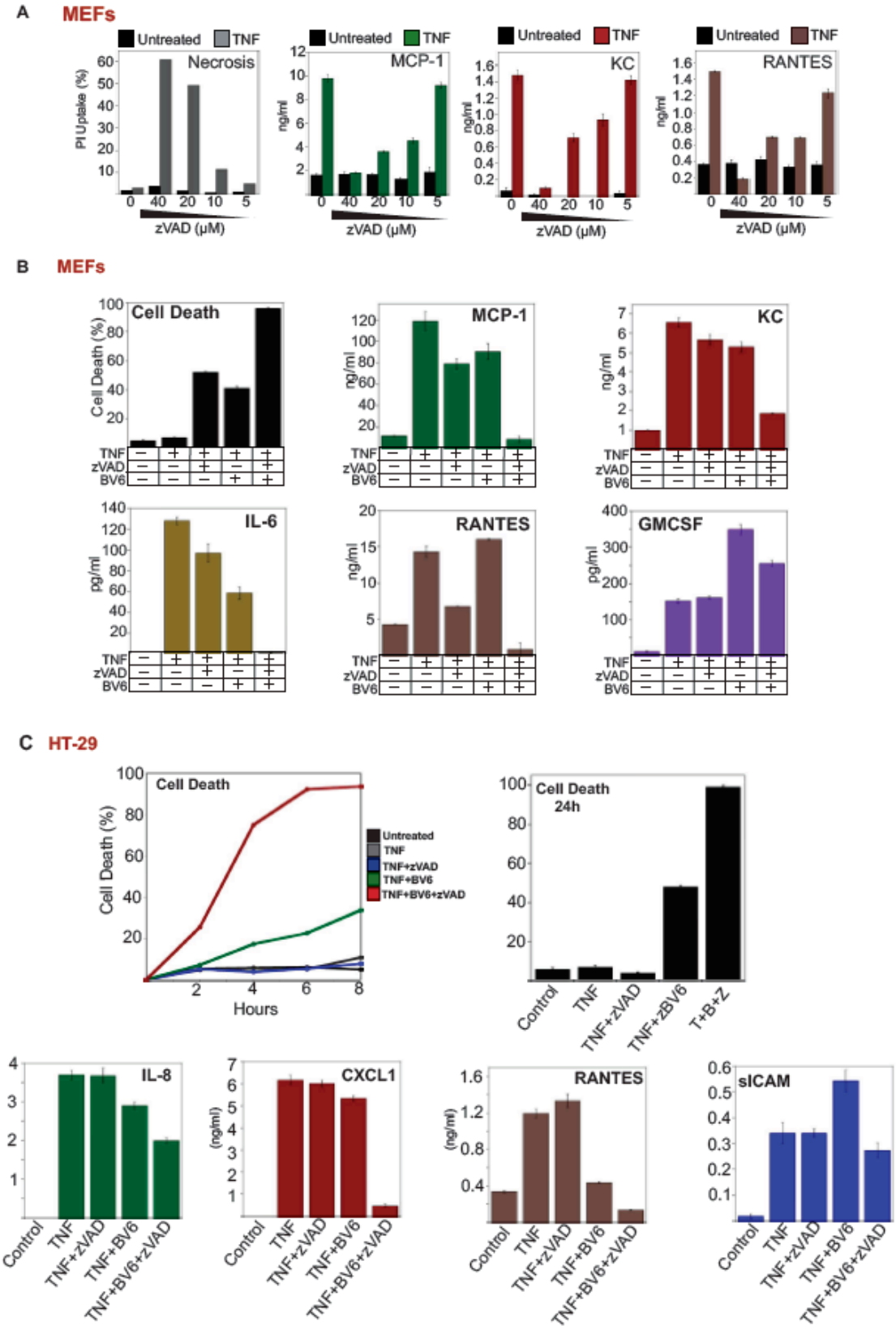

Figure S2. Necroptosis suppresses cytokine production in cells requiring sensitization.

(A) MEFs were pretreated with the indicated concentrations of zVAD for 1 h, followed by addition of TNF to 40 ng/ml. After 24 h, cell death was analysed by Propidium Iodide staining, and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA.

(B) MEFs were pretreated with BV6 (2 μM) and/or zVAD (10 μM), for 1 h, followed by addition of TNF (10 ng/ml). After 24 h, cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA.

(C) HT-29 cells were pretreated with BV6 (2 μM) and/or zVAD (10 μM), for 1 h, followed by addition of TNF (10 ng/ml). After 24 h (or the indicated timepoints), cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

Supplemental Data

Figure S3 (Related to Figure 3)

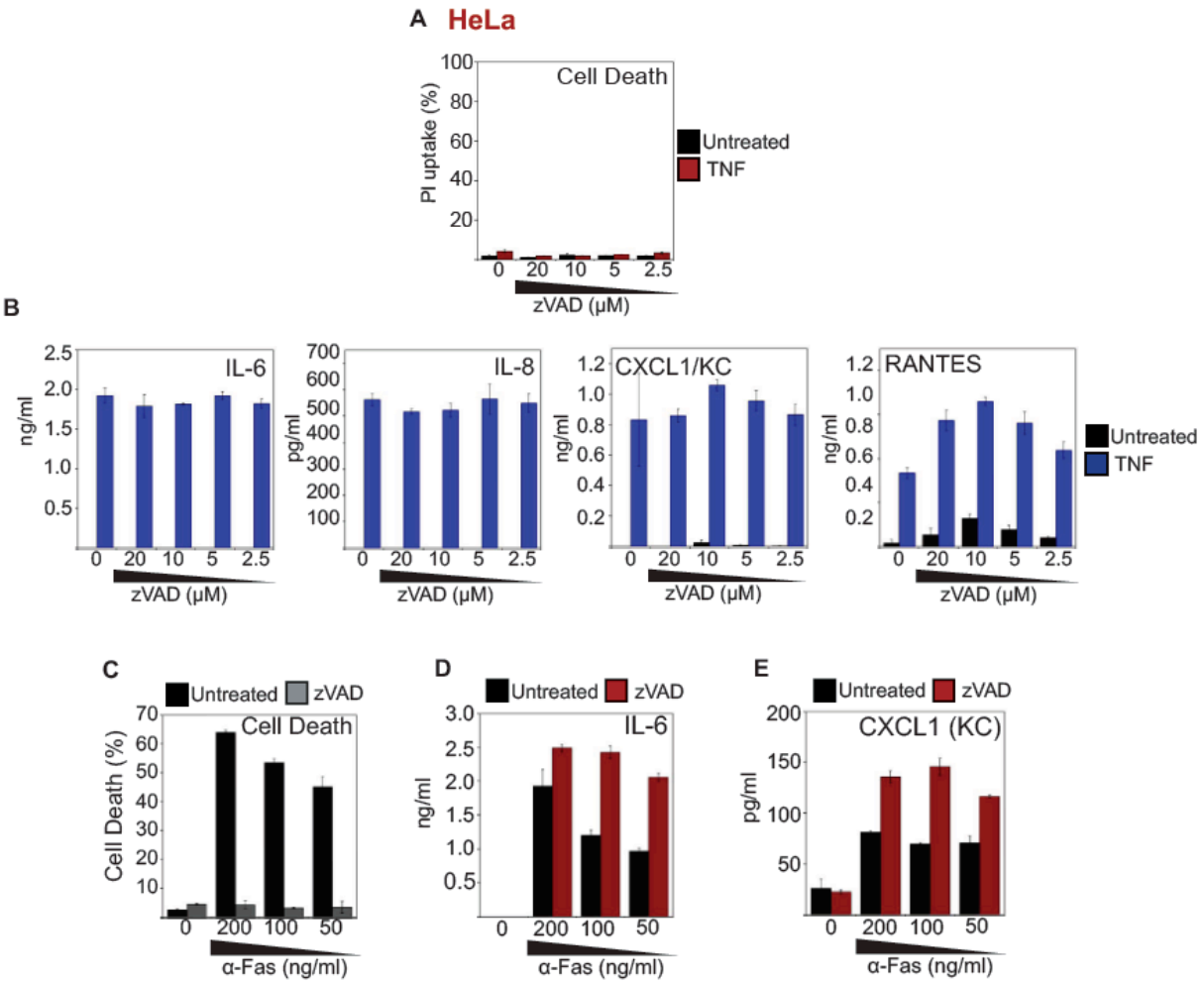

Figure S3. Caspase activity is not required for TNF-induced cytokine production.
(A) HeLa cells were pretreated with the indicated concentrations of zVAD for 1 h, followed by addition of TNF to 5 ng/ml. After 24 h, cell death was analysed by Propidium Iodide staining.
(B) Cytokine/chemokine concentrations in the culture supernatants from *A* were determined by ELISA. Error bars represent the mean ±SEM of triplicate cell cultures.
(C) HeLa cells were pretreated for 1 h in the presence or absence of zVAD (10 μM), followed by addition of anti-Fas (CH11), (100 ng/ml). After 24 h, cell death was analysed by morphology. A minimum of 300 cells was counted per treatment.
(D-E) Cytokine/chemokine concentrations in the culture supernatants from *C* were determined by ELISA. Error bars represent the mean ±SEM of triplicate cell cultures.

Supplemental Data

Figure S4 (Related to Figure 4)

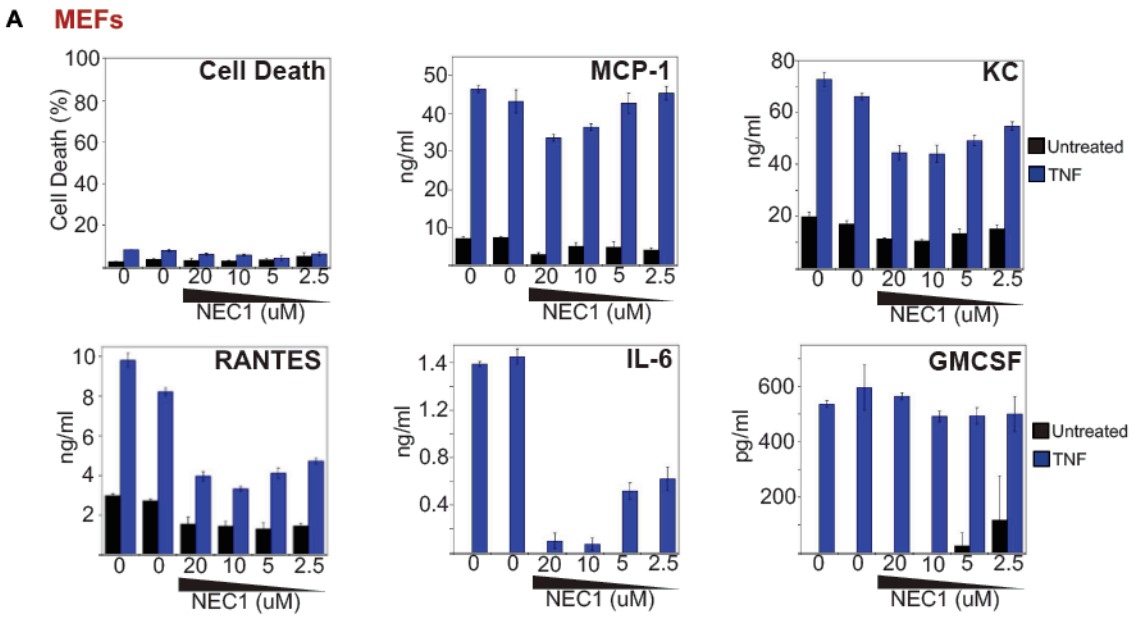

Figure S4. Nec-1 inhibits TNF-induced cytokines in MEFs.
(A) MEFs were pretreated with the indicated concentrations of Nec1 for 1 h, followed by addition of TNF (10 ng/ml). After 24 h, cell death was scored by morphology and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA. Error bars represent the mean ±SEM of triplicate determinations from a representative experiment.

Supplemental Data

Figure S5 (Related to Figure 5)

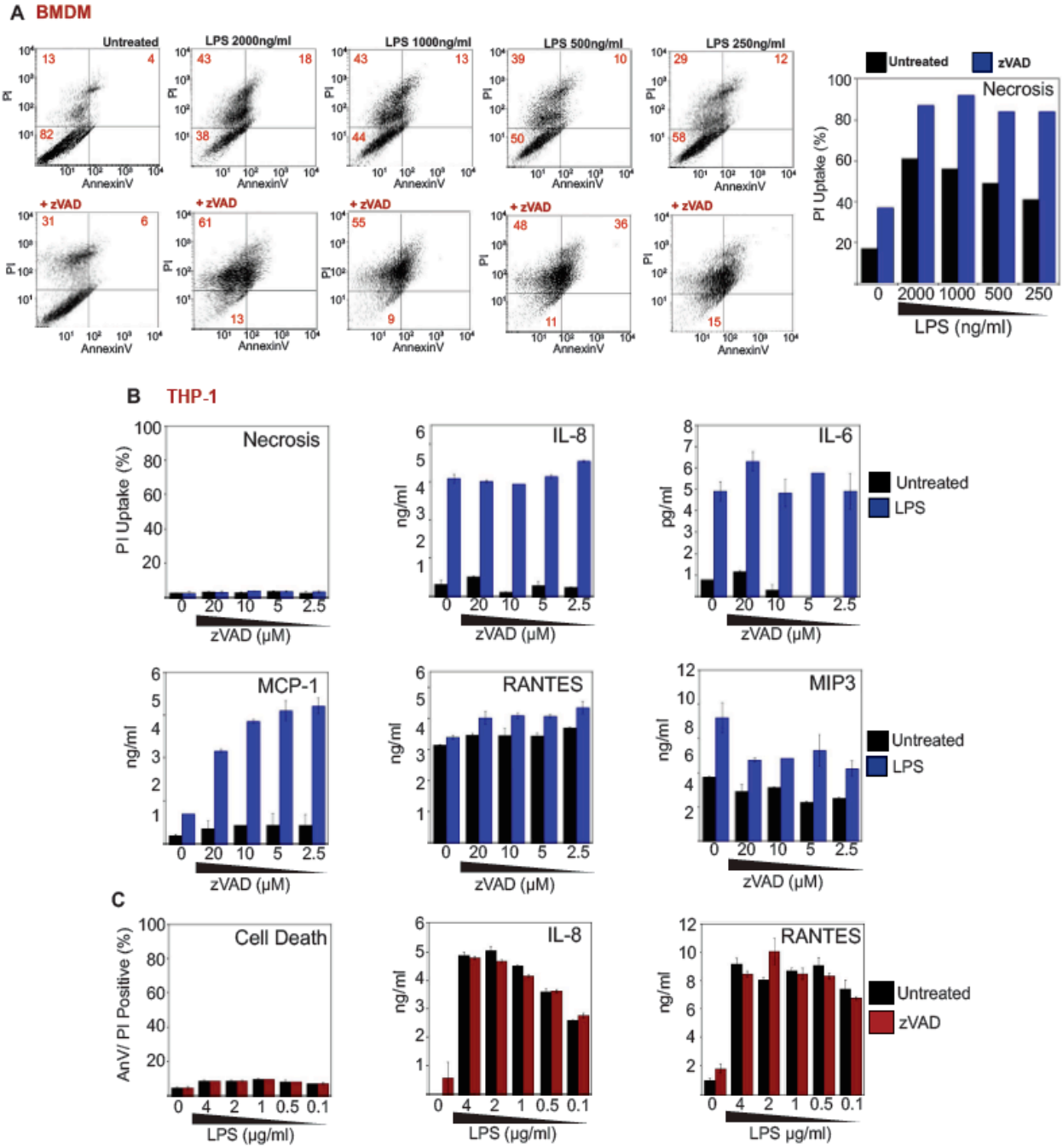

Figure S5. LPS induced necroptosis in the presence of zVAD.
(A) BMDMs were pretreated with zVAD (15 μM) for 1 h, followed by addition of LPS to the indicated concentrations. After 48 h, cell death was analysed by AnnexinV/Propidium Iodide staining.
(B) THP-1 cells were pretreated with the indicated concentrations of zVAD for 1 h, followed by addition of LPS to 5 ug/ml. After 6 h, cell death was analysed by PI uptake and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA.
(C) THP-1 cells were pretreated with zVAD (10 μM) for 1 h, followed by addition of LPS to the indicated concentrations. After 24 h, cell death was analysed by PI uptake and cytokine/chemokine concentrations in the culture supernatants were determined by ELISA.
Error bars represent the mean ±SEM of triplicate determinations from a representative experiment

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Cell culture. HT-29 cells were cultured in DMDM media (Gibco), supplemented with 10% fetal calf serum (FCS). THP-1 cells were cultured in RPMI media (Gibco) supplemented with 10 % FCS. Cells were cultured at 37°C in humidified atmosphere with 5% CO_2.

Real-Time PCR primers

Gene	Forward Primer 5'-3'	Reverse Primer 5'-3'
MCP-1	CAGCCAGATGCAATCAATGCC	5'-TGGAATCCTGAACCCACTTCT
IL-6	GAACTCCTTCTCCACAAGCGCCTT	5'-CAAAAGACCAGTGATGATTTTCACCAGG
CXCL1	AACCGAAGTCATAGCCACAC	5'-GTTGGATTTGTCACTGTTCAGC
RANTES	TACACCAGTGGCAAGTGCTC	5'-GAAGCCTCCCAAGCTAGGAC
GMCSF	GAGCATGTGAATGCCATCCAGGAG	5'-AGGTGGCGTAGAACGCGGTA
Actin	ATGTTTGAGACCTTCAACAC	5'-CACGTCACACTTCATGATGG