JEM

Supplemental material

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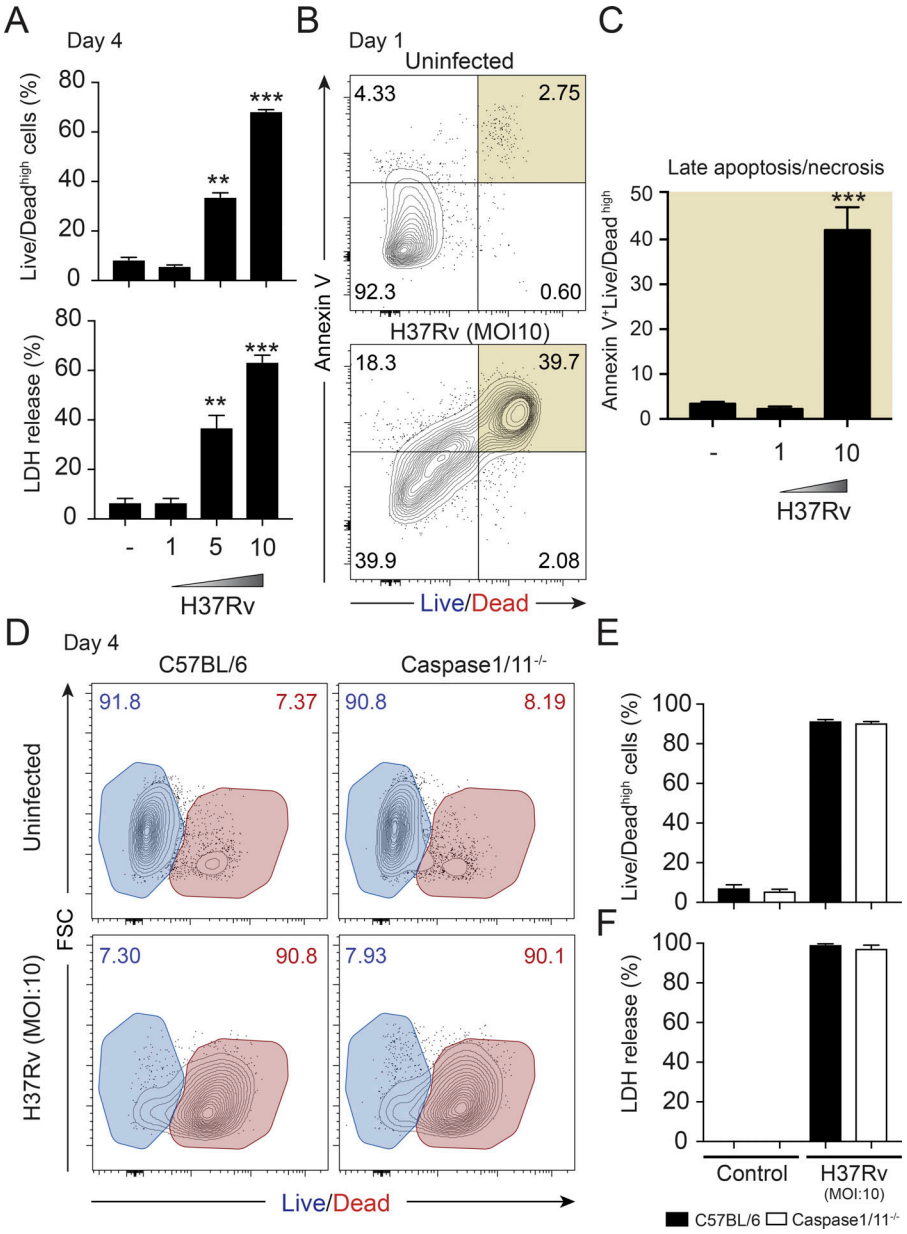

Figure S1. **The cell death occurring in Mtb-infected macrophages is nonapoptotic and caspase-1/11 independent**. C57BL/6 BMDMs were infected with H37Rv Mtb at different MOI as indicated. **(A)** Necrotic cell death was assessed by measuring Live/Dead staining (top) and by detecting LDH released (bottom) in the supernatants from the macrophage cultures at day 4 p.i. The data reported are representative results from two independent experiments performed. Statistical significance was assessed by one-way ANOVA analysis comparing infected samples with uninfected ones (**, $P < 0.01$; ***, $P < 0.001$). **(B)** Cells stained with annexin V and Live/Dead dye followed by flow cytometric analysis to detect apoptotic cells by the criteria of annexin V single positivity. Sample FACs plot demonstrating annexin V (y axis) and Live/Dead staining (x axis) in Mtb-infected and uninfected macrophage cultures at day 1 p.i. **(C)** Summary graph of the frequency of double positive annexin V and Live/Dead stained cells in macrophages infected at MOI of 1 or 10 as indicated. Data shown represent the means ± SEM of triplicate samples analyzed. Statistical significance was assessed by one-way ANOVA analysis comparing infected samples with uninfected ones (***, $P < 0.001$). Results are representative of two independent experiments performed. **(D–F)** Sample FACS plots of C57BL/6 and caspase-1/11$^{-/-}$ macrophage cultures infected with H37Rv at MOI of 10 demonstrating Mtb-induced cellular necrosis as measured by Live/Dead staining at day 4 p.i. **(E)** Summary graph of data shown in D presenting the means ± SEM of triplicate samples analyzed. **(F)** Necrotic cell death measured by LDH released in the supernatants from the macrophage cultures in D and E. The data reported in D–F are representative of two independent experiments performed. Data shown are the means ± SEM of triplicate samples analyzed.

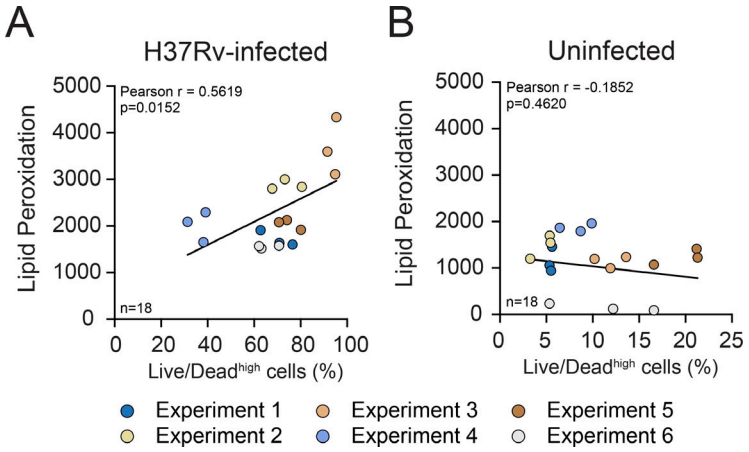

Figure S2. **The cell death occurring in Mtb-infected macrophages correlates with lipid peroxidation. (A and B)** Correlation between lipid peroxidation (LAA levels) and Live/Dead^{high} cell frequencies in Mtb-infected (A) or uninfected (B) macrophage cultures. Data shown are from six pooled independent experiments.

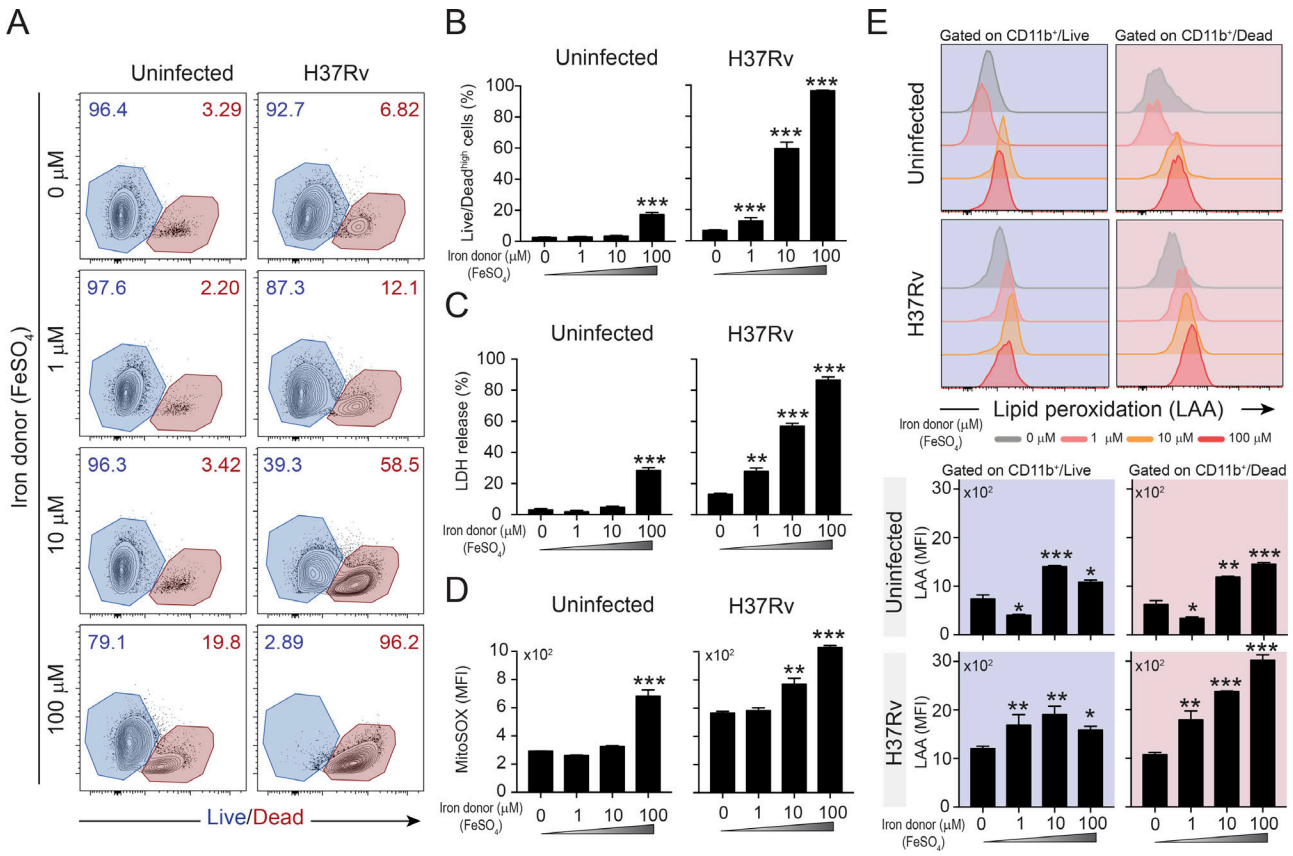

Figure S3. **Iron supplementation promotes necrotic cell death of macrophages infected with a low dose of Mtb while enhancing superoxide production and lipid peroxidation.** C57BL/6 BMDMs were infected with H37Rv Mtb at an MOI of 1 and cultures supplemented with different concentrations of an iron donor ($FeSO_4$) as indicated. On day 1 p.i., necrotic cell death measurements were performed. **(A and B)** Sample FACS plots demonstrating iron-induced macrophage necrosis in vitro as measured by Live/Dead staining. Iron concentration is indicated on the y axis and live/dead staining shown on the x axis. **(B)** Summary graph of data shown in A indicating the means ± SEM of triplicate samples analyzed. **(C)** Necrotic cell death measured by LDH release in the macrophage cultures shown in A and B. The data reported in A–C are representative results from three independent experiments performed. **(D)** Mitochondrial superoxide was quantified by MitoSOX staining and analyzed by flow cytometry. Results are representative of at least two separate experiments performed. **(E)** Lipid peroxidation in Live/Dead^{low} and Live/Dead^{high} cells measured by flow cytometry. Histograms of LAA staining are shown on the top and the summary data are presented on the bottom. Results are representative of at least three separate experiments performed. Asterisks indicate the statistical differences observed between iron-supplemented and unsupplemented cells (*, P < 0.05; **, P < 0.01; ***, P < 0.001). The data represent the means ± SEM of samples in triplicate. Results are representative of two independent experiments.

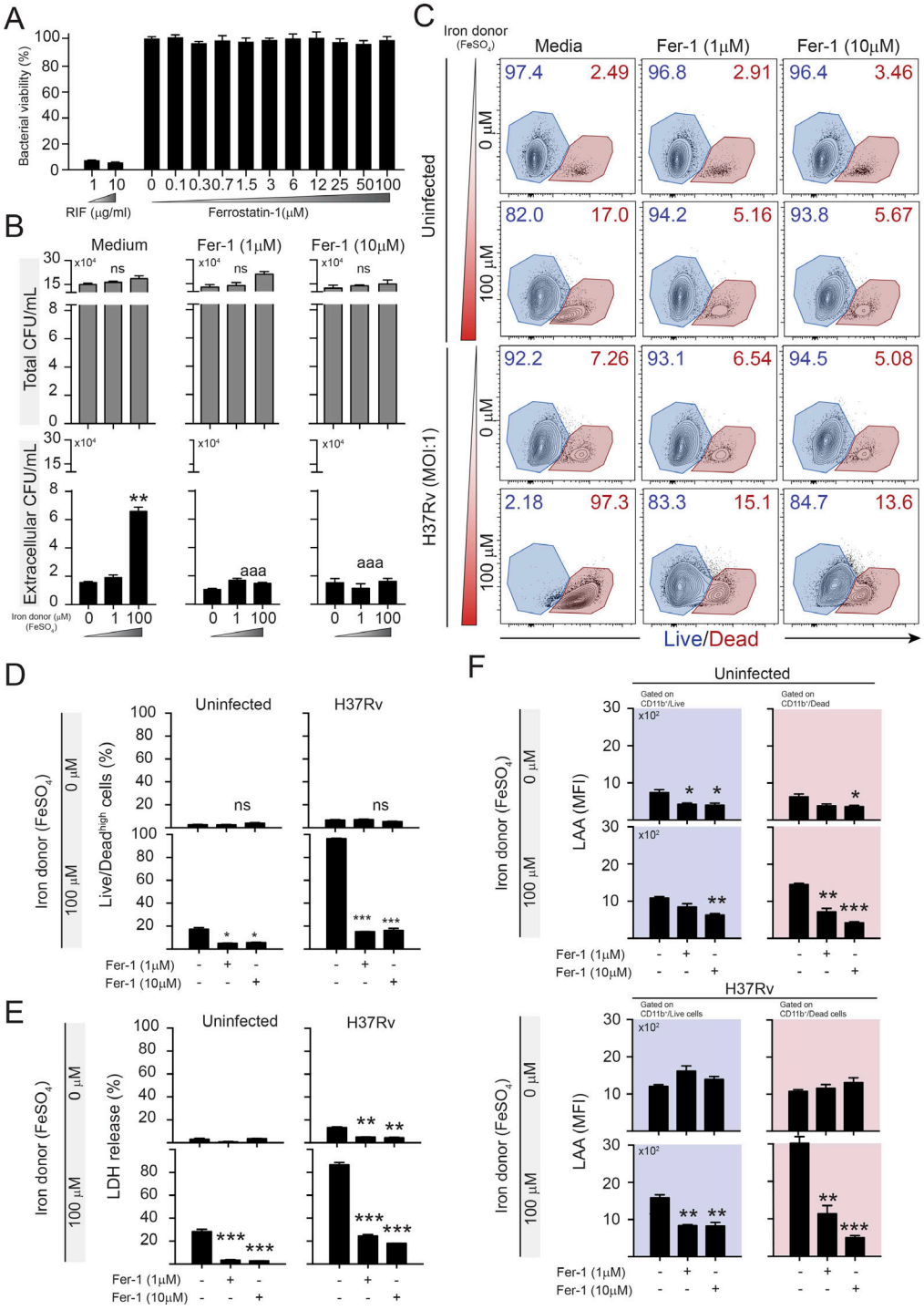

Figure S4. **Fer-1 inhibits necrotic cell death under conditions of iron supplementation. (A)** H37Rv was grown in Middlebrook 7H9 enriched with OADC and treated with different concentrations of Fer-1 for 5 d. Bacterial viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Addition of the antibiotic rifampicin was used as a positive control for mycobacterial killing. **(B–F)** C57BL/6 BMDM were infected with H37Rv Mtb at an MOI of 1 and the cultures supplemented with different concentrations of an iron donor ($FeSO_4$) as indicated. The same macrophages were treated or not with different concentrations of Fer-1 as indicated and the following measurements performed at 24 h p.i. **(B)** Mycobacterial burden evaluated by counting total CFU (top), as well as extracellular CFU as a readout of bacterial spread (bottom). **(C and D)** Sample FACS plots demonstrating Fer-1 effects on iron-induced macrophage necrosis in vitro measured by Live/Dead staining. Iron concentration is indicated on the y axis and Live/Dead staining on the x axis. **(D)** Summary graph of data shown in C presenting the means ± SEM of triplicate samples analyzed. **(E)** Necrotic cell death measured by LDH release in the macrophage cultures shown in C and D. The data shown in C–E are representative results from three independent experiments performed. **(F)** Lipid peroxidation in Live/Dead^{low} and Live/Dead^{high} cells was measured by flow cytometry at 24 h p.i. Histograms of LAA staining are shown on the top and the summary data are presented on the lower panels. Results are representative of three separate experiments performed. Asterisks indicate the statistical differences observed between Fer-1–treated and untreated macrophage cultures (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant). The data represent the means ± SEM of triplicate samples analyzed.

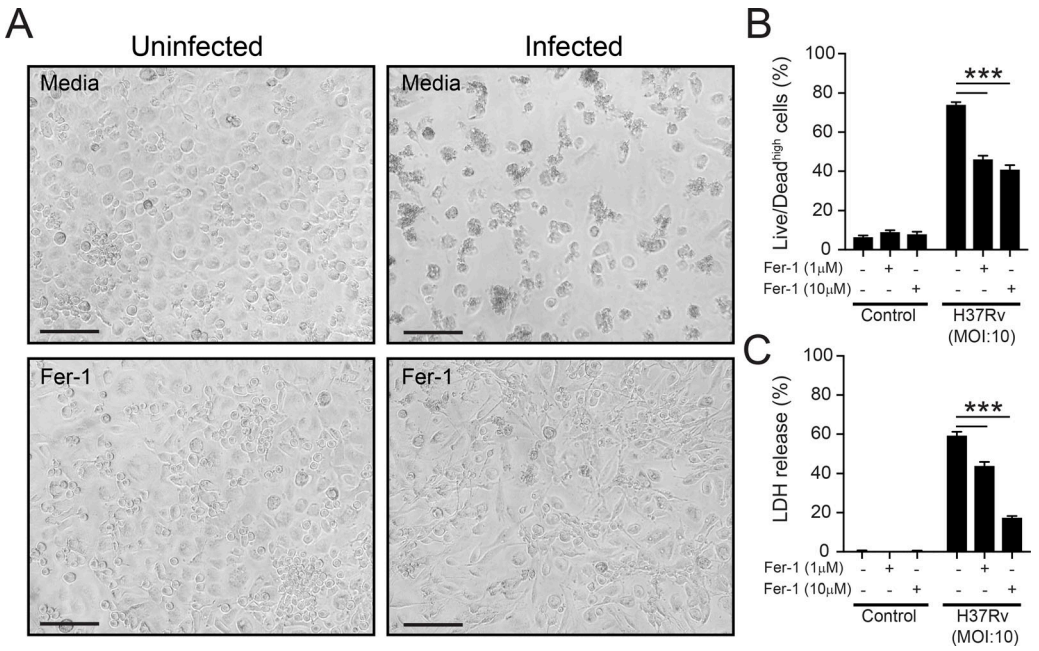

Figure S5. **Fer-1 inhibits Mtb-induced necrotic cell death in human monocyte–derived macrophages.** Human monocyte–derived macrophages were infected or not with H37Rv Mtb at an MOI of 10. Macrophage cultures were treated with Fer-1 at different concentrations indicated. Necrotic cell death was evaluated on day 4 p.i. **(A)** Representative images of uninfected or infected macrophage cultures untreated (top) or treated (bottom) with Fer-1 (10 µm) on day 4 p.i. (bars, 50 µm). **(B)** Necrosis assessed by Live/Dead staining and analyzed by flow cytometry. **(C)** LDH release measured in supernatants from macrophage cultures. The data represent the means ± SEM of triplicate samples analyzed.