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Supplemental Information

A Two-Cell Model for IL-1β Release Mediated by Death-Receptor Signaling

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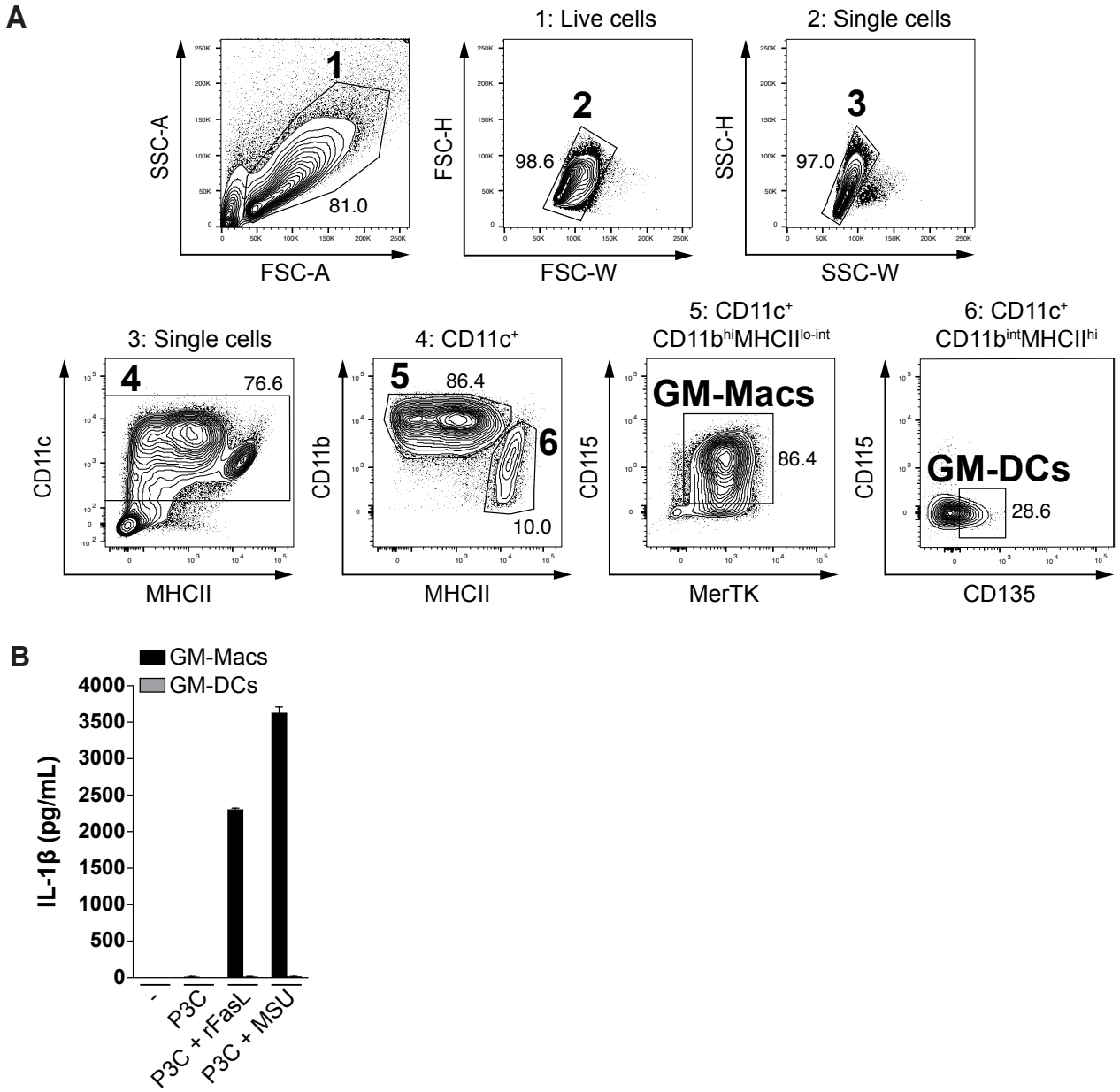

Figure S1 (related to Figure 2): Monocyte-derived Macrophages and not DCs are Responsible for FasL-induced IL-1β Release in GM-CSF Mouse Bone Marrow Cultures

(A) Representative flow cytometry plots demonstrating the gating strategy utilized to FACS-sort GM-Macs (GM-CSF monocyte-derived macrophages, $CD11c^{+}CD11b^{hi}MHCII^{lo\text{-}int}CD115^{+}MerTK^{+}$) and GM-DCs (GM-CSF-derived DCs, $CD11c^{+}CD11b^{int}MHCII^{hi}CD115^{-}CD135^{+}$) from mouse bone marrow cultures grown for 7 days in GM-CSF-containing media. Boxes depict gates (labeled with large, bold text) and the small numbers correspond to percentage of cells in each gate. The parent gates are labeled at the top of each plot.

(B) GM-Macs and GM-DCs were FACS-sorted from seven-day GM-CSF bone marrow cultures and were left untreated or primed with P3C for 6 hours prior to stimulation with rFasL for 24 hours. IL-1β release was quantified by ELISA. MSU-treated P3C-primed GM-Macs or GM-DCs were used as a control.

Error bars represent mean + SD of triplicate samples. Each panel is representative of two different experiments.

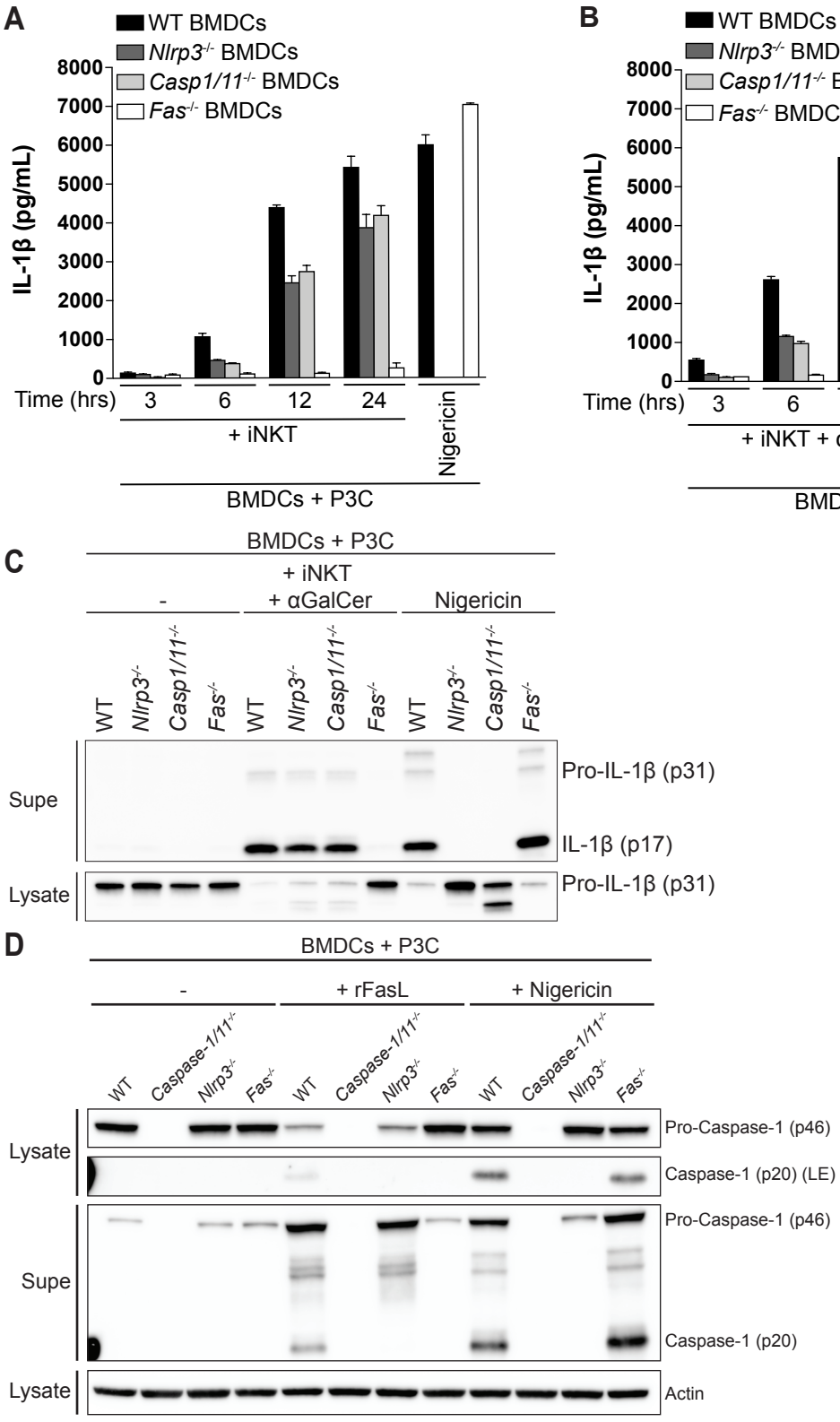

Figure S2 (related to Figure 3): Fas Ligation Elicits Inflammasome-dependent and -independent IL-1β Release by TLR-Primed BMDCs

(A-C) P3C-primed BMDCs from WT, *Nlrp3*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Fas*$^{-/-}$ mice were cultured alone or with iNKT cells for (A and B) the indicated times or (C) 12 hours in the (A) absence or (B and C) presence of αGalCer. (A and B) IL-1β release was quantified by ELISA and (C) cell-associated and extracellular IL-1β were analyzed by immunoblot. Nigericin-treated BMDCs were used as a control.

(D) P3C-primed BMDCs from WT, *Nlrp3*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Fas*$^{-/-}$ mice were stimulated with rFasL for 6 hours and the cell lysates and cell-free supernatants were probed for the indicated proteins by immunoblot.

Error bars represent mean + SD of triplicate samples. Each panel is representative of at least three different experiments.

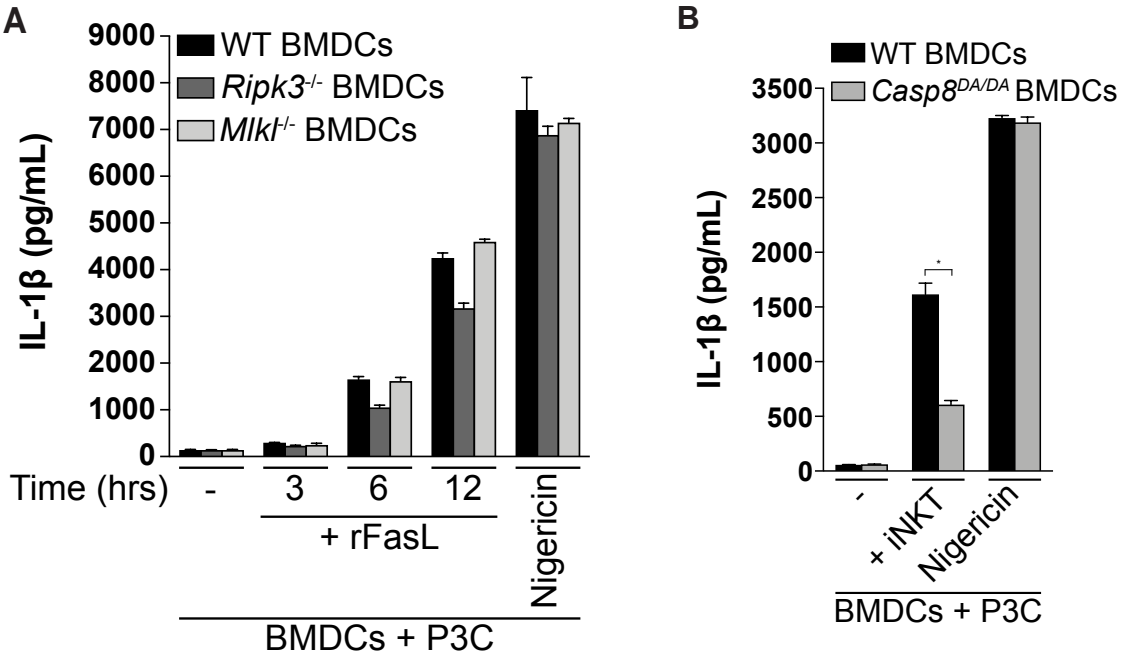

Figure S3 (related to Figure 3): FasL-Induced IL-1β Release by BMDCs Proceeds Independently of Necroptosis and Requires Caspase-8 Self-Cleavage

(A) P3C-primed BMDCs from WT, *Ripk3*$^{-/-}$, and *Mlkl*$^{-/-}$ mice were cultured alone or in the presence of rFasL for the indicated times. IL-1β release was quantified by ELISA. Nigericin-treated BMDCs were used as a control.

(B) P3C-primed BMDCs from WT and *Caspase-8*$^{DA/DA}$ mice were cultured alone or with iNKT cells for 24 hours. IL-1β release was quantified by ELISA.

Error bars represent mean + SD of triplicate samples. Each panel is representative of at least three different experiments. *p <0.05 as determined by Student's unpaired t test.

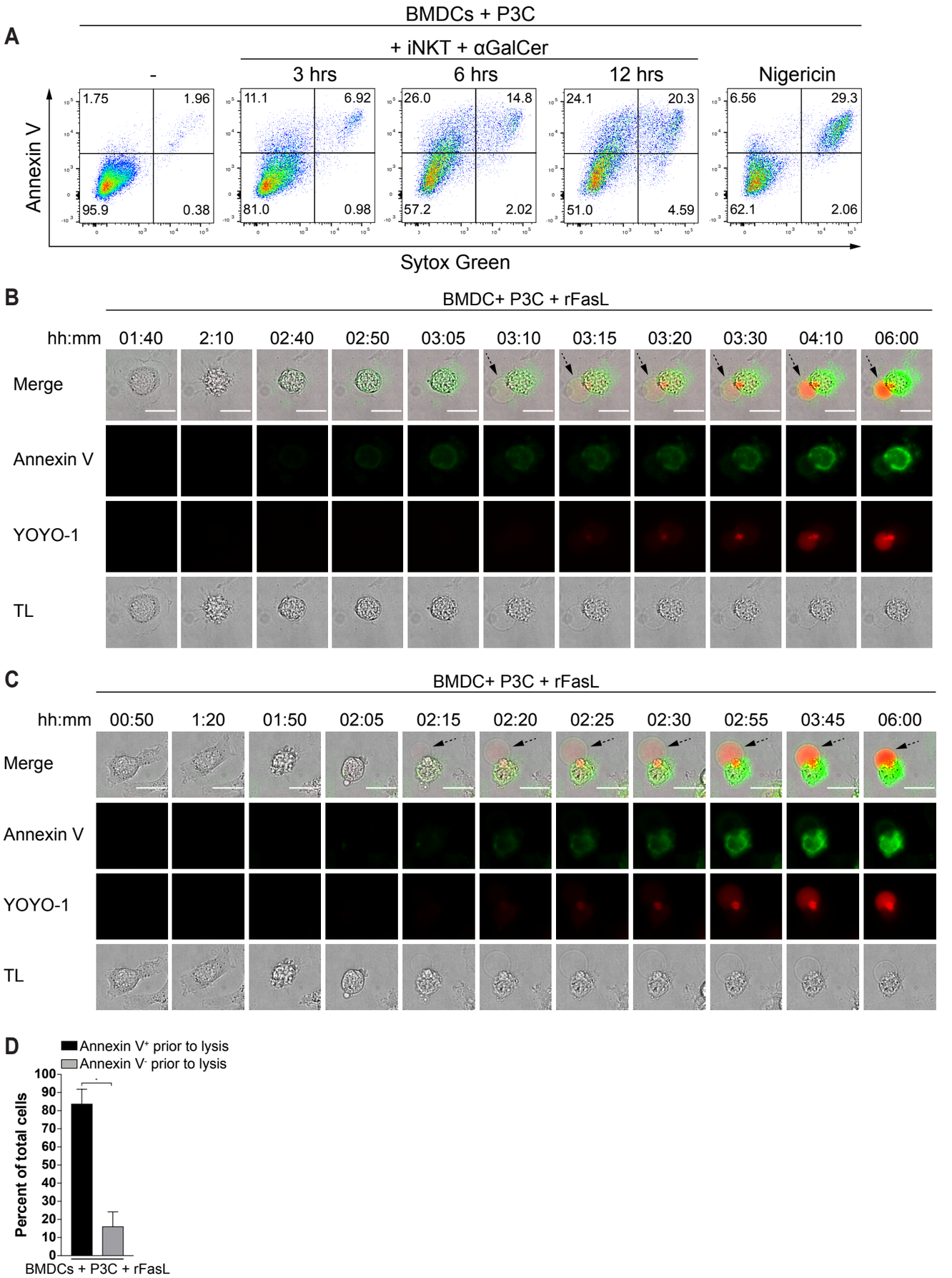

Figure S4 (related to Figure 5): Fas Ligation Elicits Apoptosis that Rapidly Transitions to Necrosis in TLR-primed BMDCs

(A) P3C-primed BMDCs were cultured alone or with CellTrace Violet-labeled iNKT cells in the presence of αGalCer. After the indicated times, the cells were harvested, labeled with Sytox Green and annexin V and the extent of early-apoptotic and necrotic BMDCs was analyzed by gating on the CellTrace Violet-negative, $CD11c^+$ cells. Nigericin-treated BMDCs were used as a control.

(B and C) P3C-primed BMDCs were stimulated with rFasL and morphological changes, annexin V (green) and YOYO-1 (red) staining were monitored by live imaging over 6 hours. Images were captured every 5 minutes and representative timepoints are shown. Dotted arrows point at necrotic plasma membrane balloons. The scale bars represent 10 μm. Note that while the cell in (B) stains with annexin V prior to the formation of necrotic balloons, the cell in (C) becomes annexin V-positive following lysis.

(D) P3C-primed BMDCs were stimulated with rFasL for 6 hours and the number of cells that stained positive for annexin V prior (Annexin V^+ prior to lysis) or concurrently (Annexin V^- prior to lysis) to the formation of necrotic plasma membrane balloons was quantified. A total of 893 cells were counted.

Error bars represent mean + SD of 27 replicate wells from two different experiments. Panels A, B, and C are representative of at least three different experiments. *p <0.05 as determined by Student's unpaired t test.

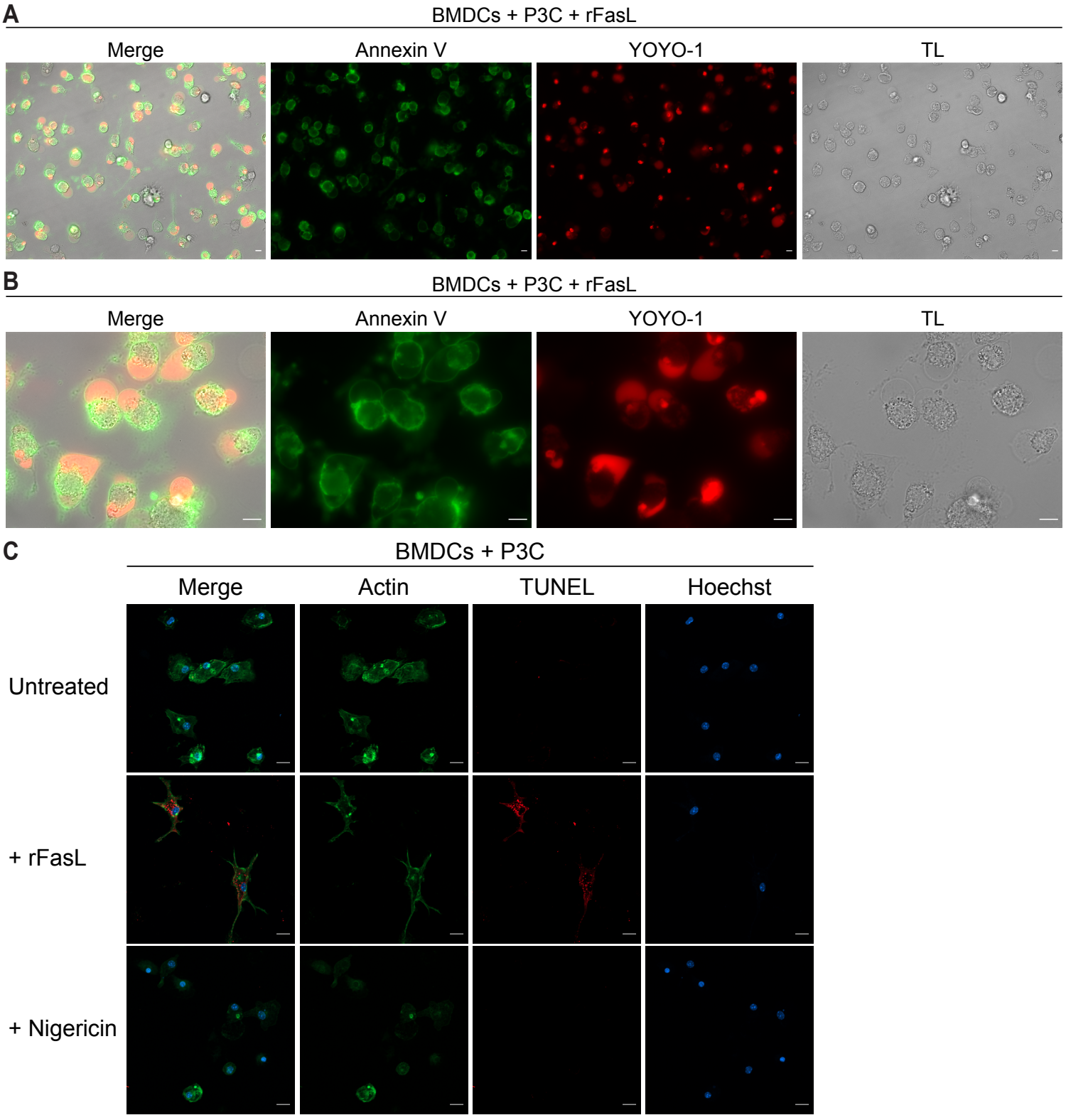

Figure S5 (related to figure 6): Nuclear DNA diffuses to the Cytosol and Necrotic Balloons when Necrosis is Preceded by Apoptosis in TLR-Primed BMDCs

(A and B) P3C-primed BMDCs were stimulated with rFasL and morphological changes (TL), annexin V (green) and YOYO-1 (red) staining were assessed by live imaging after 4 hours. Representative images demonstrate the extent of YOYO-1 diffusion to the cytosol and necrotic plasma membrane balloons following Fas ligation at (A) 20X and (B) 63X magnification. The scale bars represent 10 μm.

(C) P3C-primed BMDCs were left untreated or stimulated with rFasL or nigericin for 6 hours or 1 hour, respectively. TUNEL staining (red) was performed to assess the extent of DNA fragmentation. Actin (green) and nuclear (Hoechst, blue) staining are also shown. The scale bars represent 20 μm.

Each panel is representative of at least three different experiments.

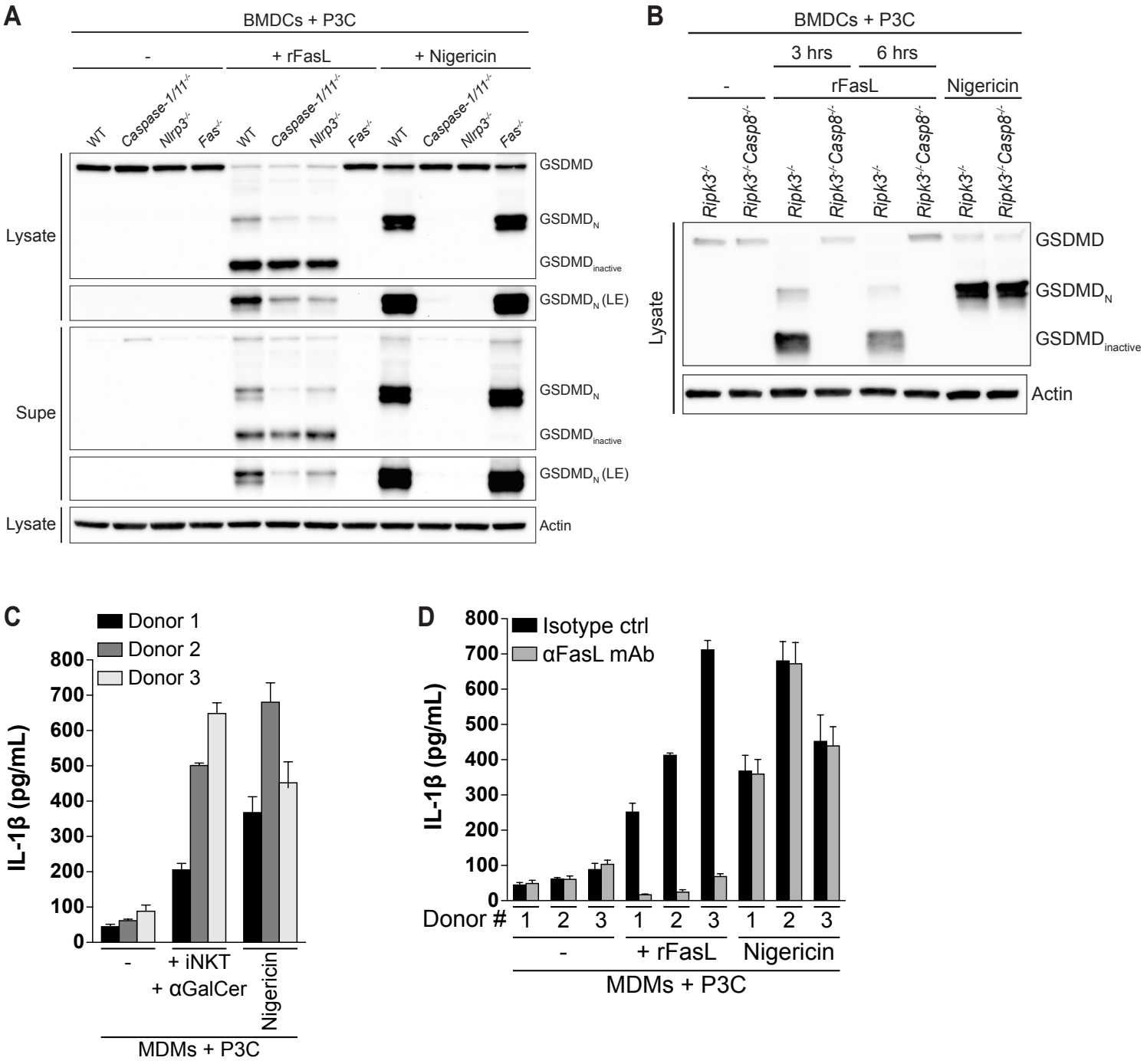

Figure S6 (related to figure 7): Caspase-8 Mediates Inflammasome-independent Cleavage of GSDMD in response to Fas ligation

(A and B) P3C-primed BMDCs from WT, *Nlrp3*$^{-/-}$, *Caspase1/11*$^{-/-}$ and *Fas*$^{-/-}$ mice or (B) *Ripk3*$^{-/-}$ and *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice were stimulated with rFasL for (A) 6 hours or (B) the indicated times and the cell lysates and cell-free supernatants were probed for the indicated proteins by immunoblot.

(C) P3C-primed human monocyte-derived macrophages (MDMs) from 3 donors were cultured alone or with iNKT cells for 24 hours in the presence of αGalCer (50 ng/ml). IL-1β release was quantified by ELISA. As a control, MDMs were treated with nigericin (10 μM) for 2 hours.

(D) P3C-primed MDMs from 3 donors were stimulated with rFasL for 24 hours in the presence of a FasL-blocking antibody (αFasL mAb) or an isotype control. IL-1β release was quantified by ELISA.

Panels are representative of at least three different experiments. LE = long exposure, Supe = supernatant.

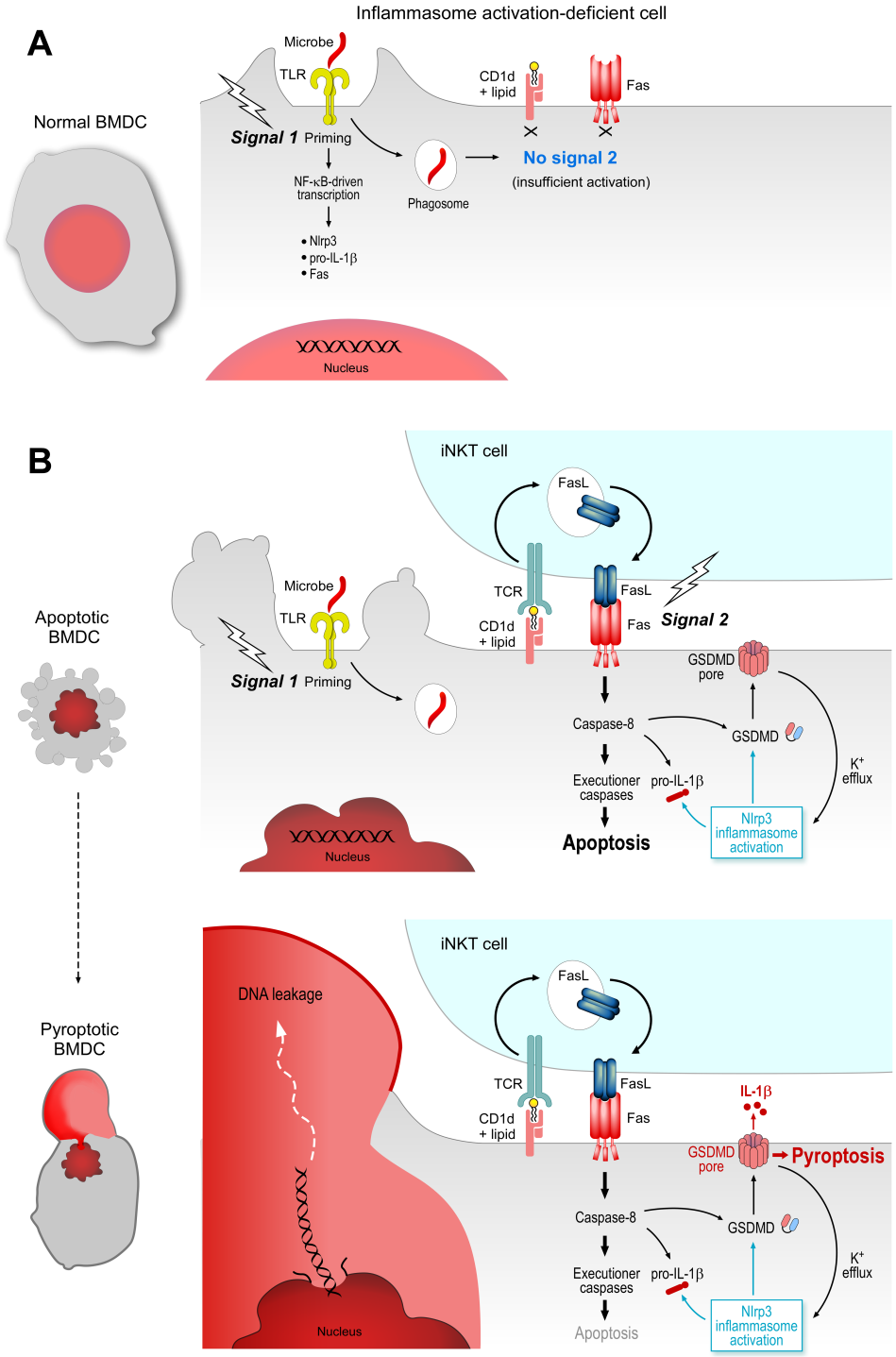

Figure S7 (related to figures 1, 2, 3, 4, 5, 6, and 7): Model for iNKT Cell-Induced IL-1β Release by TLR-Primed BMDCs

(A) Microorganisms that stimulate a TLR on a BMDC will provide it with an inflammasome priming signal, but they may fail to provide an inflammasome activating signal from within the cytosol of the host cell.

(B) BMDCs exposed to microbes can activate iNKT cells through the presentation of stimulatory self-lipid antigens on CD1d. In turn, the activated iNKT cell rapidly translocates intracellular FasL to the surface, ligates Fas on the BMDC, and activates a caspase-8-driven signaling module that initiates the apoptotic program and drives IL-1β processing and release in an inflammasome-dependent and -independent manner. Through caspase-8-mediated activation of the pore-forming activity of GSDMD, Fas ligation mediates potassium efflux, resulting in the activation of the Nlrp3 inflammasome. GSDMD mediates the switch from apoptosis to pyroptosis, resulting in the lytic, inflammatory death of the BMDCs characterized by the release of nuclear DNA into the cytoplasm and pyroptotic plasma membrane balloons. This two-cell model for IL-1β release can potentially trigger IL-1β release during infection with any microorganism that stimulates a TLR on the BMDC, even if the microbe fails to elicit cell-autonomous inflammasome activation.