SUPPLEMENTAL MATERIAL

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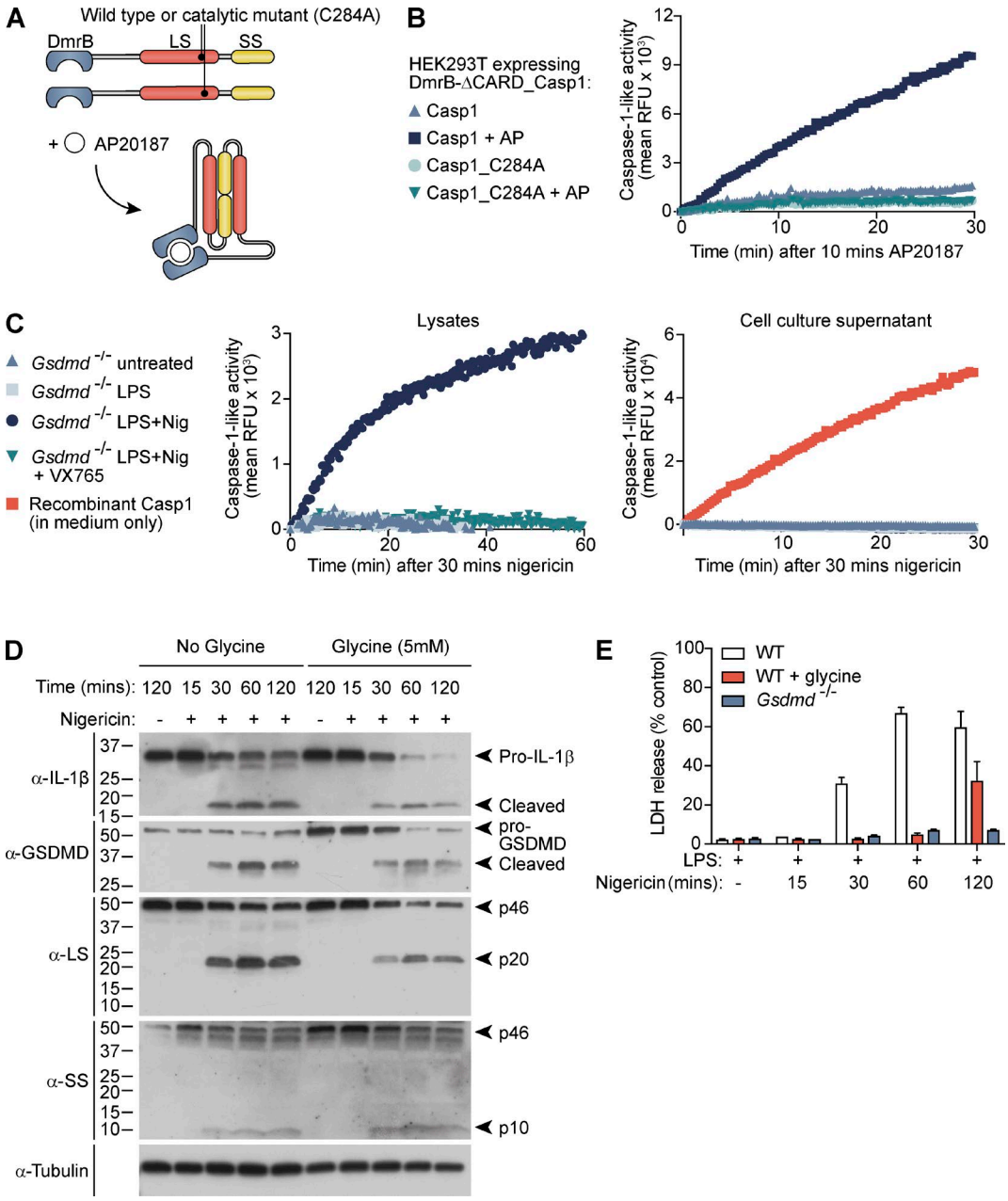

Figure S1. **Cellular caspase-1 activity is induced by dimerization and terminated in a manner independent of GSDMD or frank cell lysis. (A and B)** An inducible dimerizer system depicted in A was used to demonstrate that dimerization induces caspase-1 proteolytic activity (B). HEK293T were transfected with plasmids encoding DmrB-ΔCARD-caspase-1 (WT vs. catalytic mutant, C284A). 18 h after transfection, cells were replated for assay. 4 h later, the DmrB dimerizer drug (AP20187 [AP], 1 µM) was added to cells to induce dimerization of DmrB-ΔCARD-caspase-1. 10 min later, cellular caspase-1–like activity was quantified over time. **(C)** *Gsdmd*$^{-/-}$ macrophages were left untreated or LPS-primed for 4 h and stimulated with nigericin for 30 min (±10 µM VX765 added 30 min before nigericin). Caspase activity in cell lysates or cell culture medium was then quantified by incubating these fractions with caspase assay buffer containing the caspase-1 fluorogenic substrate, YVAD-afc, and monitoring caspase-1–like activity over a further 30–60 min. Caspase-1–like activity of *Gsdmd*$^{-/-}$ macrophages stimulated with LPS + nigericin declined over time (as observed by the loss of linearity of the activity over time), indicating protease inactivation. Recombinant ΔCARD–caspase-1 (50 nM; p20/p10) in cell culture medium was assayed in parallel as a positive control for caspase activity in full medium and to demonstrate that the YVAD-afc substrate is not limiting in the assay. **(D and E)** LPS-primed WT and *Gsdmd*$^{-/-}$ macrophages were stimulated with nigericin for the indicated times. Cells were exposed to 5 mM glycine or left untreated, 30 min before nigericin addition. Cell culture supernatants and extracts were harvested. (D) WT cell supernatants were precipitated and resuspended in cell extracts, and the kinetics of caspase-1 substrate cleavage (pro–IL-1β cleavage to p17, pro-GSDMD to GSDMD p30) was examined by Western blot. (E) Cell culture supernatants were assayed for LDH release as an indicator of cell lysis. Graphs are mean of triplicate cell samples (B and C) or mean + range of duplicate cell samples from a single experiment (E). All data are representative of at least three (B) or four (C–E) independent experiments.

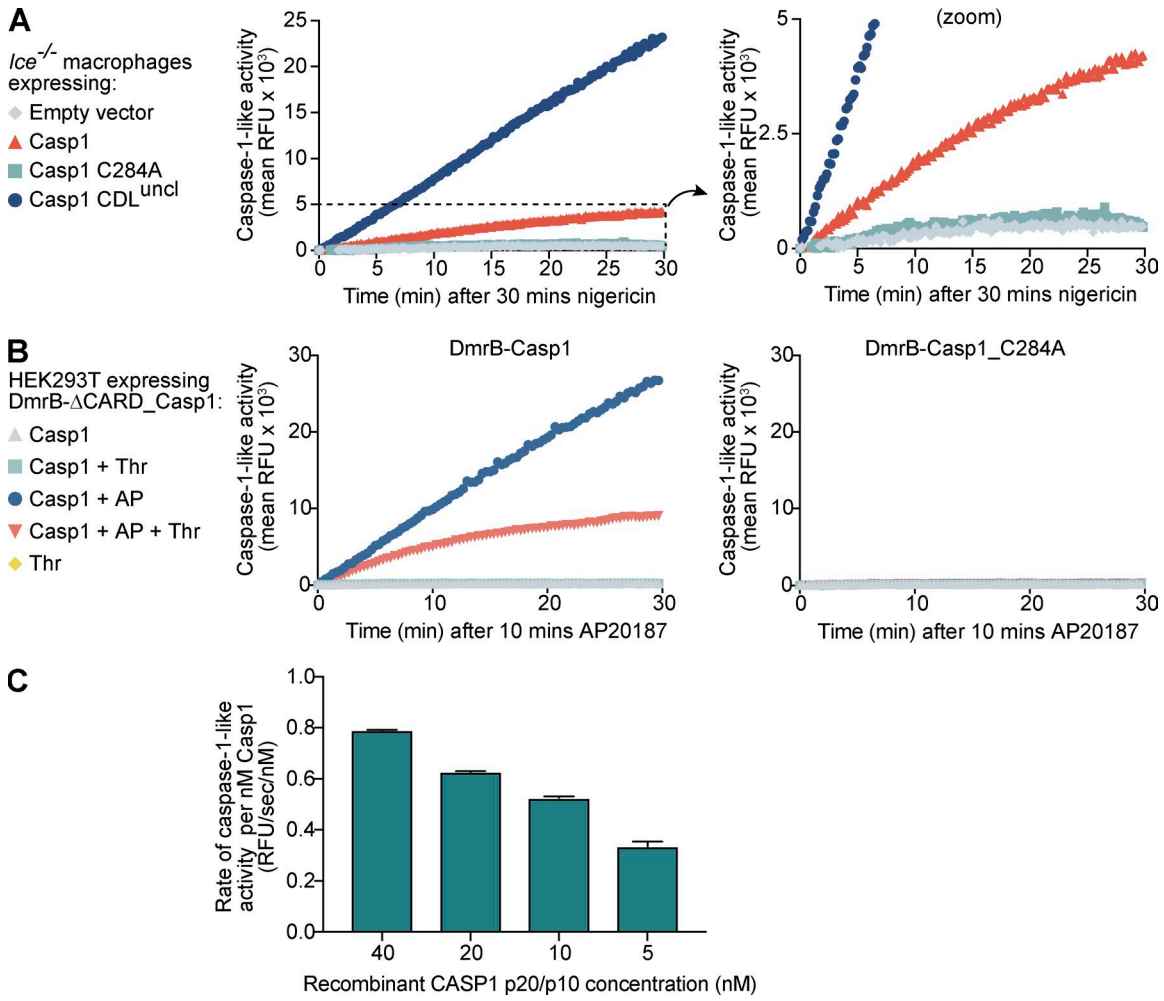

Figure S2. **Caspase–1 cleavage at the CDL in cis or trans inactivates proteolytic activity, and dilution of recombinant caspase–1 p20/p10 triggers its loss of relative activity. (A and B)** Caspase-1 constructs engineered to prevent self-cleavage at the CDL (Casp1 CDL^{uncl}; A) or to allow CDL cleavage in trans by thrombin (B) were expressed in *Ice*$^{-/-}$ macrophages (A) or HEK293T (B). In A, macrophages were LPS primed and stimulated with nigericin for 30 min; in B, transfected HEK293T were treated with 1 µM DmrB dimerizer drug, AP20187 for 10 min. The cell culture supernatant was then removed, and cells were assayed for caspase-1–like activity. Where appropriate, thrombin was added to the assay reaction at the same time as the caspase activity buffer. Notably, CDL^{uncl} caspase-1 (A) and AP20187-dimerized caspase-1 unable to self-process (B) displayed linear activity profiles, unlike WT caspase-1 (A) and thrombin-cleaved caspase-1 (B). **(C)** Recombinant caspase-1 p20/p10 was diluted to 5–40 nM immediately before assaying caspase-1–like activity, which was monitored over 20 min. Data are expressed as normalized rate of caspase-1–like activity per nM caspase-1 over the first 20 min after dilution. All data are mean (+SD) of triplicate samples and are representative of at least three independent experiments.

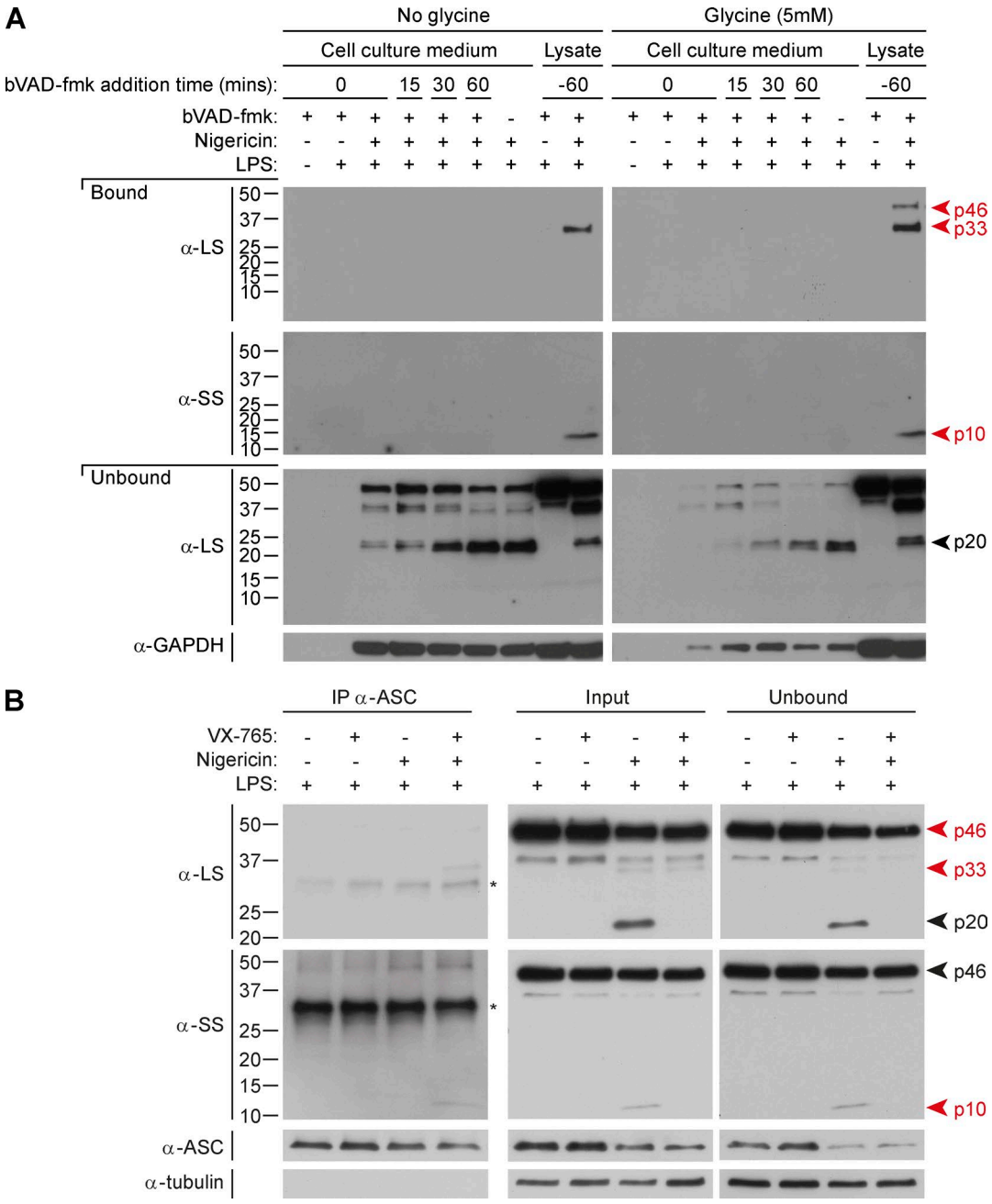

Figure S3. **Caspase-1 is active (p33/p10) only on the inflammasome, and not when released into cell culture medium (p20/p10). (A)** Macrophages were either left untreated or LPS-primed for 4 h before nigericin stimulation for a further 2 h. bVAD-fmk was applied to cells at various times after nigericin (0, 15, 30, 60 min). Cell culture medium was removed and subjected to streptavidin pull-down (shown here). Unfractionated lysate of the cells sample treated with bVAD-fmk for 60 min before treatment ± nigericin was assayed in parallel as a positive control for p33/p10 pull-down. Cells were harvested, fractionated, and analyzed in Fig. 3 A. Cells were treated with 5 mM glycine 30 min before nigericin to delay cell rupture (right). Data are representative of at least three independent experiments. **(B)** Macrophages were LPS-primed (4 h) and stimulated with nigericin for 30 min (with and without VX765 application immediately before nigericin). ASC was immunoprecipitated, and coimmunoprecipitated caspase-1 species were examined by immunoblot. *, Nonspecific band. Data are representative of four independent experiments.

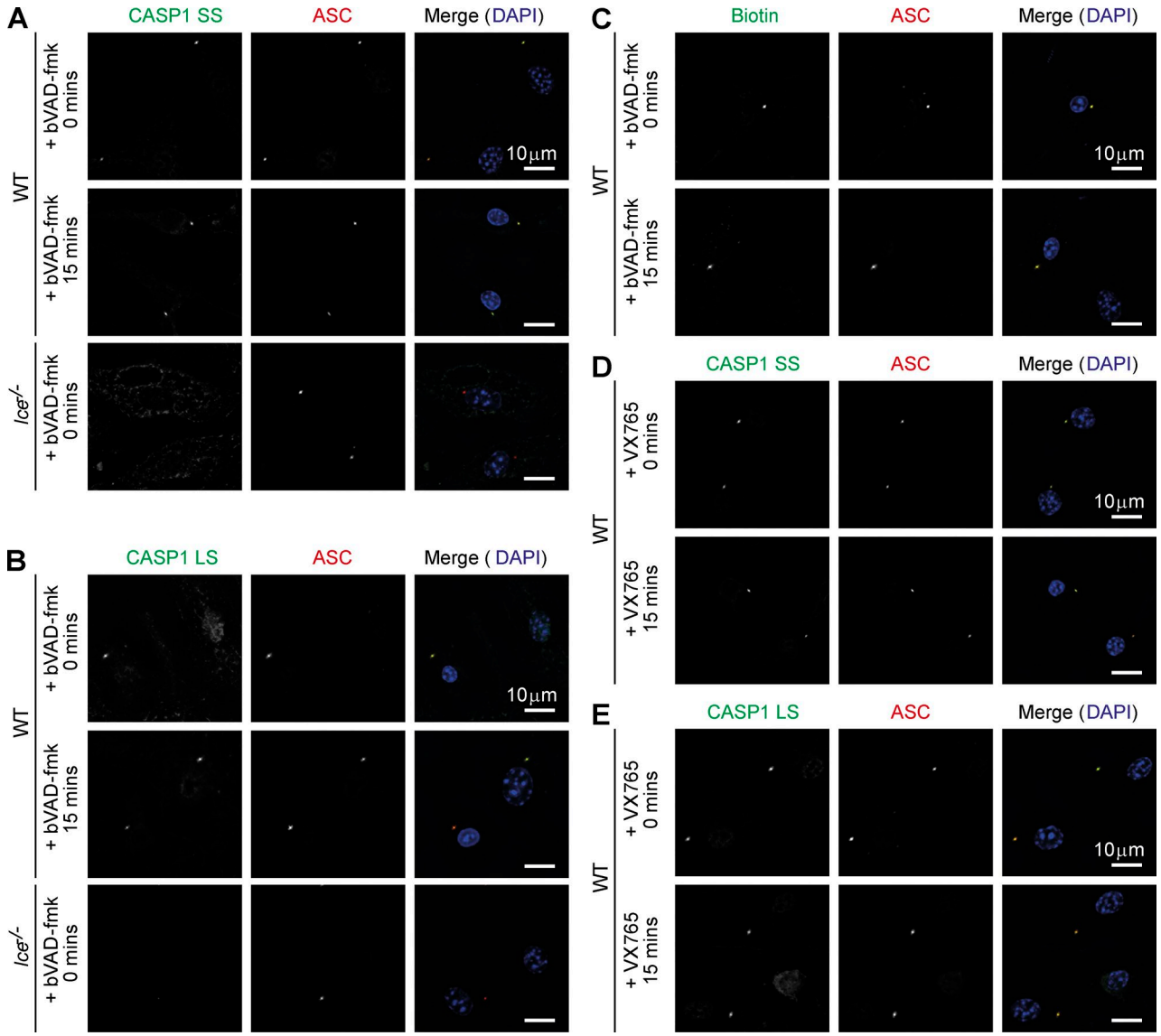

Figure S4. **Active caspase-1 colocalizes with the ASC speck in nigericin-stimulated macrophages. (A–D)** WT or caspase-1–deficient (*Ice*$^{-/-}$) macrophages were LPS-primed (4 h) and stimulated with nigericin for 30 min. Caspase-1 inhibitors (bVAD-fmk, VX765) were added at the indicated times after nigericin. Immunofluorescence labeling of ASC versus caspase-1 (small subunit; A and D), caspase-1 (large subunit; B and E), or biotin (C). Nuclei are stained with DAPI. Images are maximum-intensity projections of *z*-stacks (ImageJ) and are representative of at least three independent experiments.

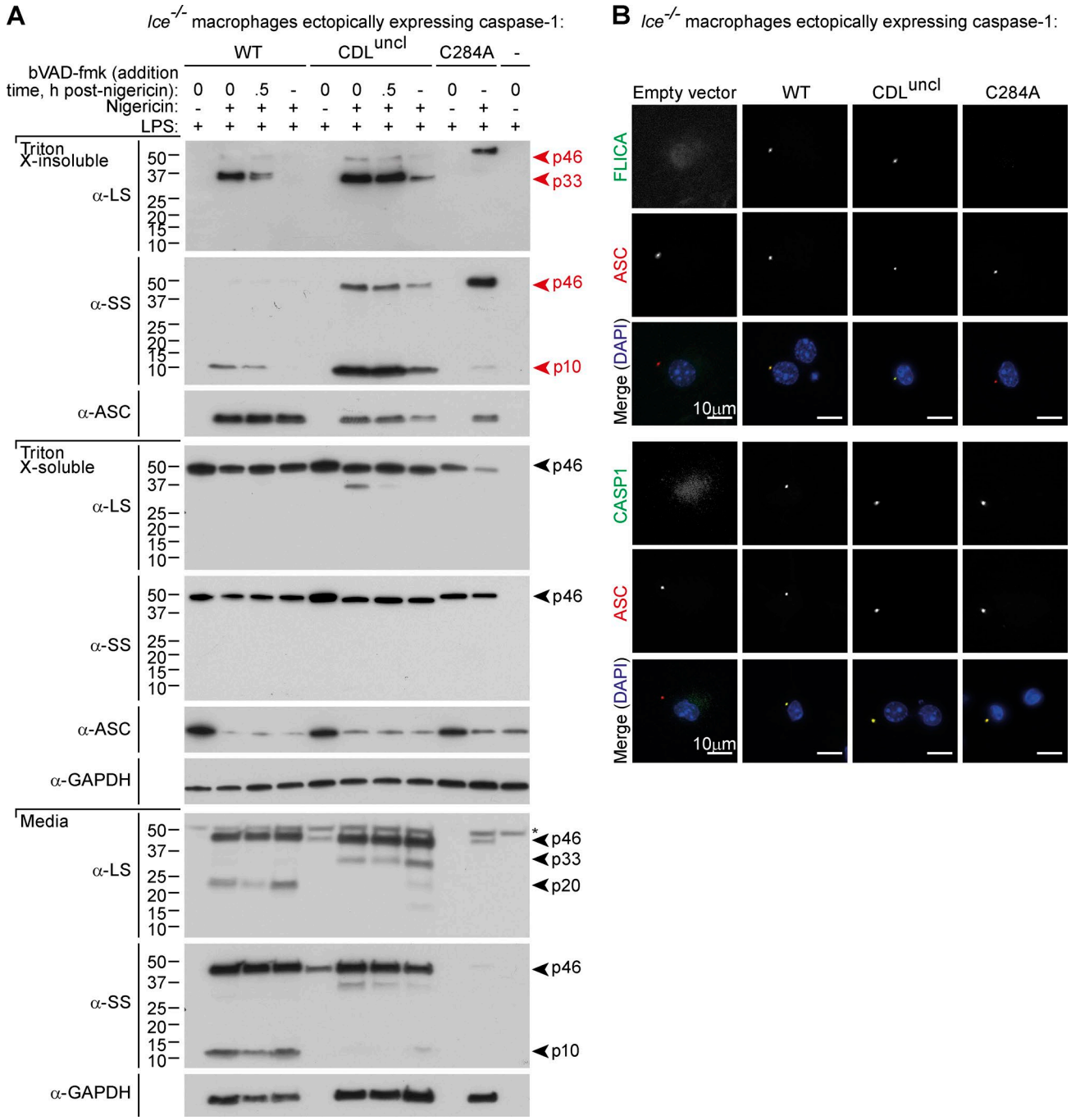

Figure S5. **A CDL-uncleavable mutant of caspase-1 localizes to the ASC speck.** Bone marrow progenitors from *Ice*$^{-/-}$ (*Casp1*$^{-/-}$/*Casp11*$^{null/null}$) mice were retrovirally reconstituted for mouse caspase-1 (WT; E102A/D103A/E121A/D122A compound mutant unable to self-process at the CDL, CDL^{uncl}; catalytic mutant C284A; vs. empty vector control, –) during their differentiation to macrophages. Differentiated, transduced macrophages were LPS-primed for 4 h, before stimulation with nigericin. **(A)** 10 µM bVAD-fmk was applied to cells at the same time as or 0.5 h after nigericin. Cells were harvested and fractionated 2 h after nigericin stimulation. The ASC speck-containing (Triton X-100–insoluble) fraction and the Triton X-100–soluble fraction were analyzed for caspase-1 species by Western blot. *, Nonspecific band. **(B)** Active caspase-1 (FAM-FLICA applied at the same time as nigericin) versus total caspase-1, localized relative to the ASC speck 30 min after nigericin stimulation. Images are maximum-intensity projections of *z*-stacks (ImageJ). All data are representative of three independent experiments.