Immunity

Supplemental Information

BNIP3- and BNIP3L-Mediated Mitophagy Promotes the Generation of Natural Killer Cell Memory

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Supplementary Figure 1

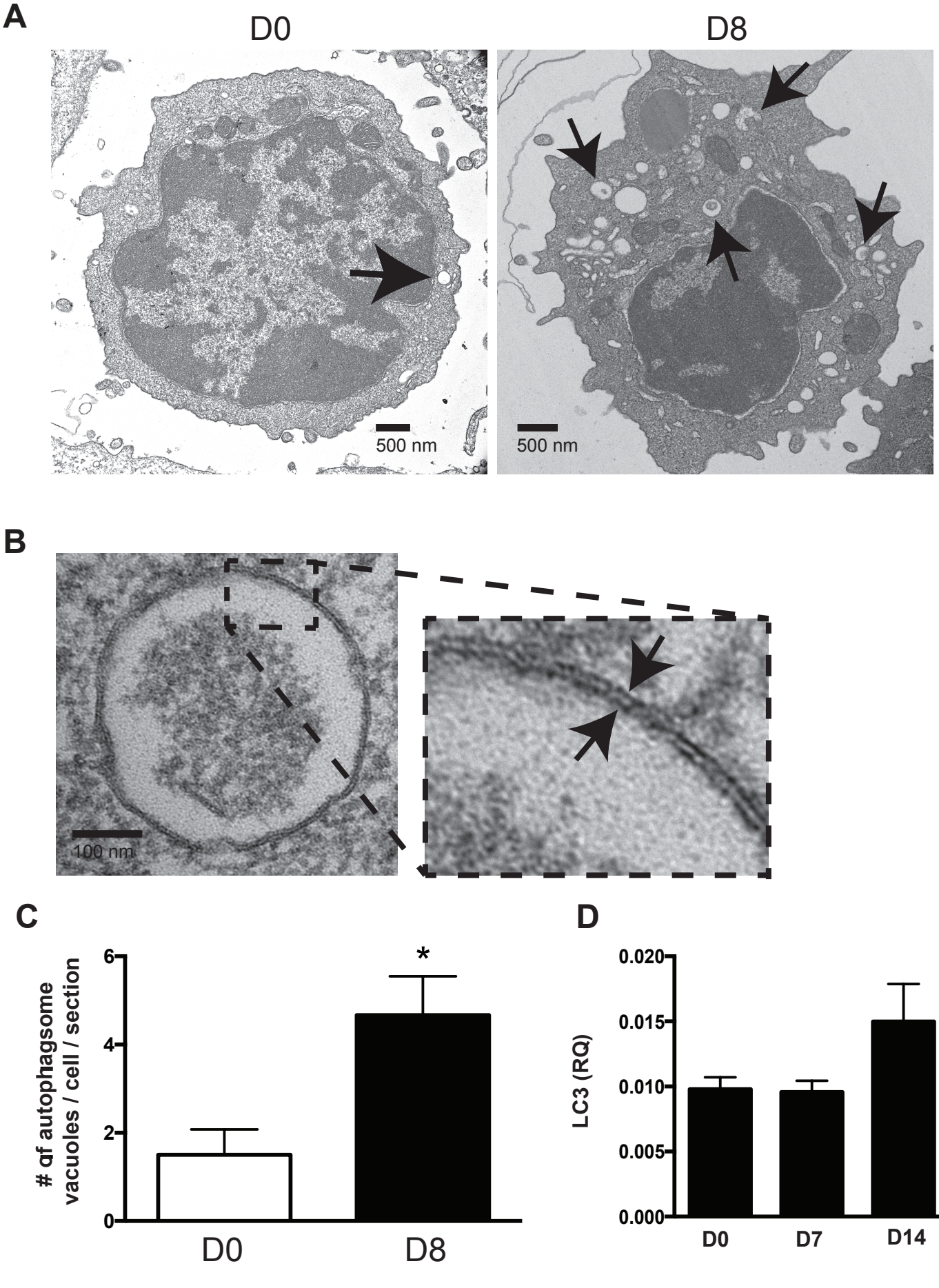

Figure S1, related to Figure 2. Analysis of autophagosome formation in effector NK cells. LC3-GFP Ly49H$^+$ NK cells were adoptively transferred into Ly49H-deficient mice and infected with MCMV. (**A**) Splenic Ly49H$^+$ NK cells were then sorted at D8 PI or D0 from LC3-GFP mice and analyzed by transmission electron microscopy (TEM). Representative TEM images of naïve (D0) or effector (D8 PI) Ly49H$^+$ NK cells at 25,000X magnification. Arrows indicate identified autophagosome structures. **(B**) Representative TEM image of a double membrane vesicle containing cytoplasmic cargo (or autophagosome) at 250,000X magnification. Arrows indicate each individual membrane. **(C**) Quantification of autophagosome structures per cell per section analyzed for each time point as indicated. **(D**) Splenic Ly49H$^+$ NK cells were sorted at the indicated time points PI from recipient mice, and analyzed for LC3b mRNA by qRT-PCR (n=3 for each time point). Data are representative of two independent experiments, with n = 3 per group per time point, or at least 4 cells/section with 4 independent sections with the indicated bar scale**.**

Supplementary Figure 2

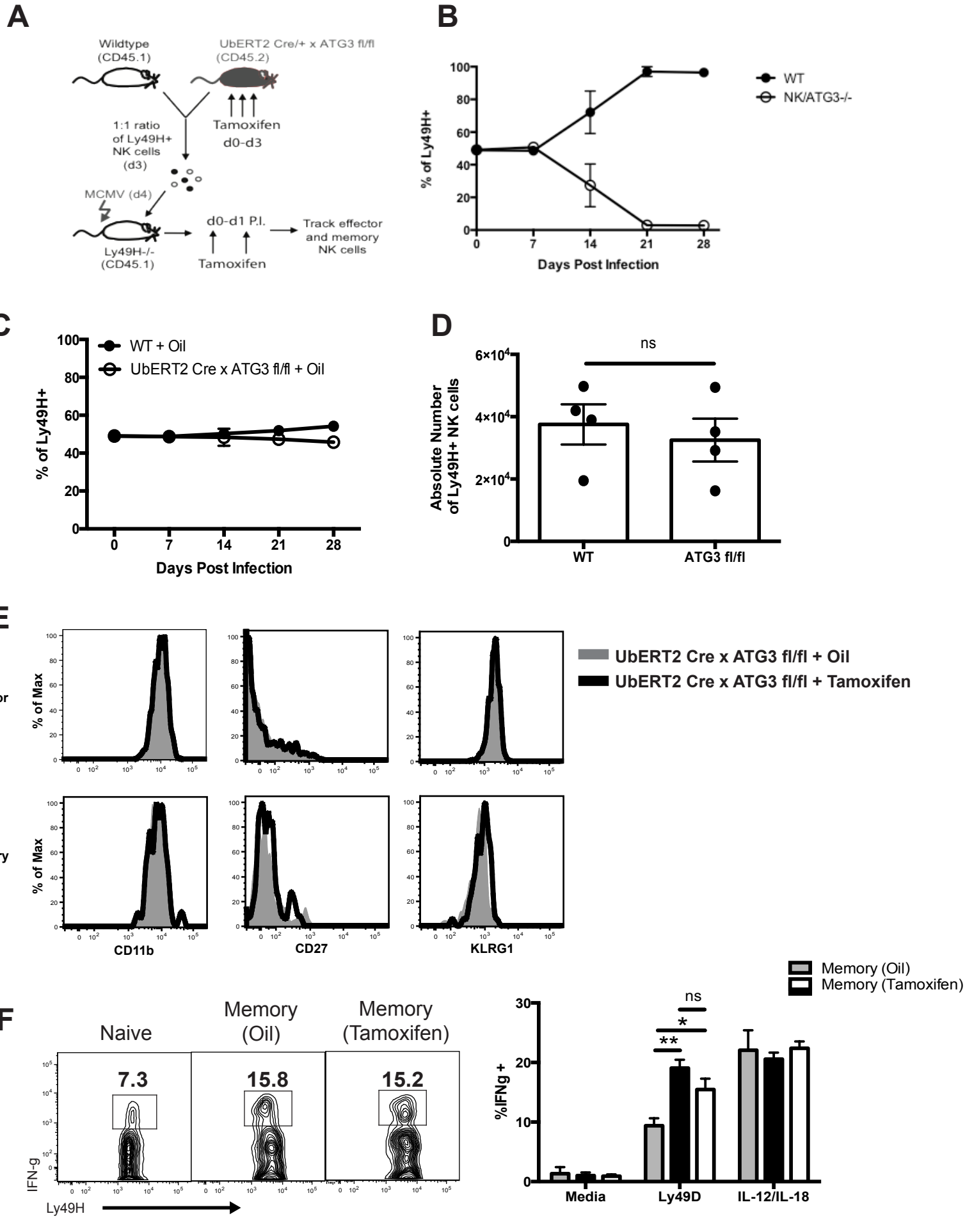

Figure S2, related to Figure 3. *Atg*3 is critical for the generation of memory NK cells. (**A**) *UbcERT2-Cre x Atg3*$^{fl/fl}$ (CD45.2) mice received 1mg of tamoxifen i.p. daily from day 0 to 3. 2 x 10^5 WT (CD45.1) and *UbcERT2-Cre x Atg3*$^{fl/fl}$ (CD45.2) splenic Ly49H$^+$ NK cells were mixed at a 1:1 ratio, transferred into Ly49H-deficient mice, and infected with MCMV. Recipients then received 1mg of tamoxifen i.p. daily from day 0 to 1 PI. (**B**) Percentages in the blood at various time points PI. 2 x 105 WT (CD45.1) and *UbcERT2-Cre x Atg3*$^{fl/fl}$ (CD45.2) splenic Ly49H$^+$ NK cells were mixed at a 1:1 ratio, transferred into Ly49H- deficient mice, and infected with MCMV. Recipients then received 200ul of corn oil i.p. daily from day 4 to 8 PI (**C**) Percentages in the blood at various time points PI and (**D**) absolute numbers of indicated adoptively transferred Ly49H$^+$ NK cells on day 28 PI from recipient spleens (**E**) Representative histograms show cell surface staining of indicated markers on adoptively transferred Ly49H$^+$ NK cell populations. (**F**) Representative plots and quantitation of intracellular IFN−γ in naïve or indicated adoptively transferred Ly49H$^+$ NK cells at D28 PI after stimulation with plate-bound anti-Ly49D monoclonal antibodies or IL-12 and IL-18 for 5 h**.**

Supplementary Figure 3

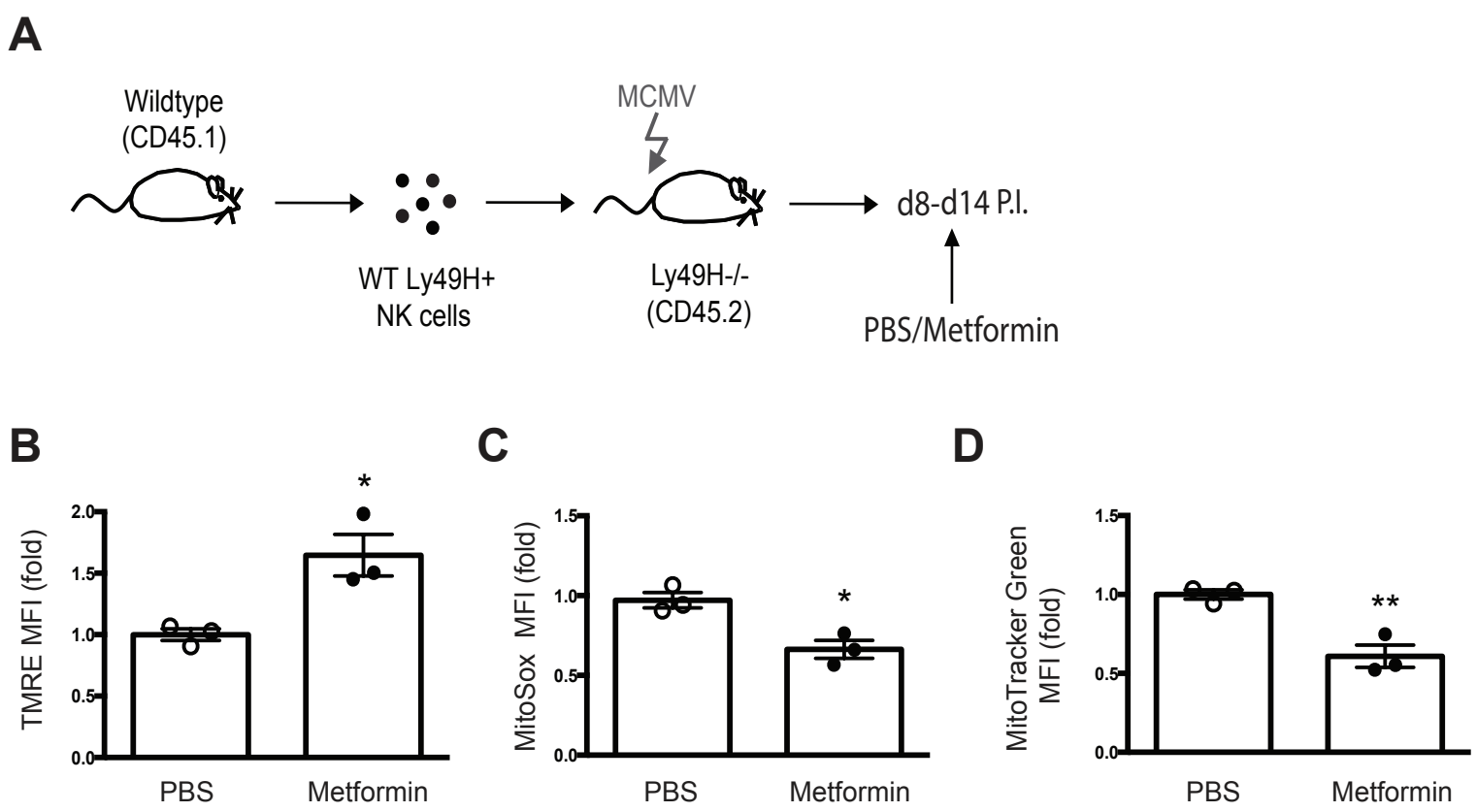

Figure S3, related to Figure 4. Metformin-induced autophagic activity enhances dysfunctional mitochondrial clearance in NK cells during the contraction phase. (A) Schematic of experiment. Briefly, splenic Ly49H$^+$ NK cells (CD45.1+) were transferred into Ly49H-deficient mice, and infected with M**CM**V. Recipient mice were then injected with either metformin or PBS i.p. daily from d8-d14 PI**. (B-D)** MitoTracker Green, TMRE, and MitoSox Red MFI values for transferred Ly49H$^+$ cells were normalized to PBS controls and plotted as the fold change in MFI at indicated time points PI.

Supplementary Figure 4

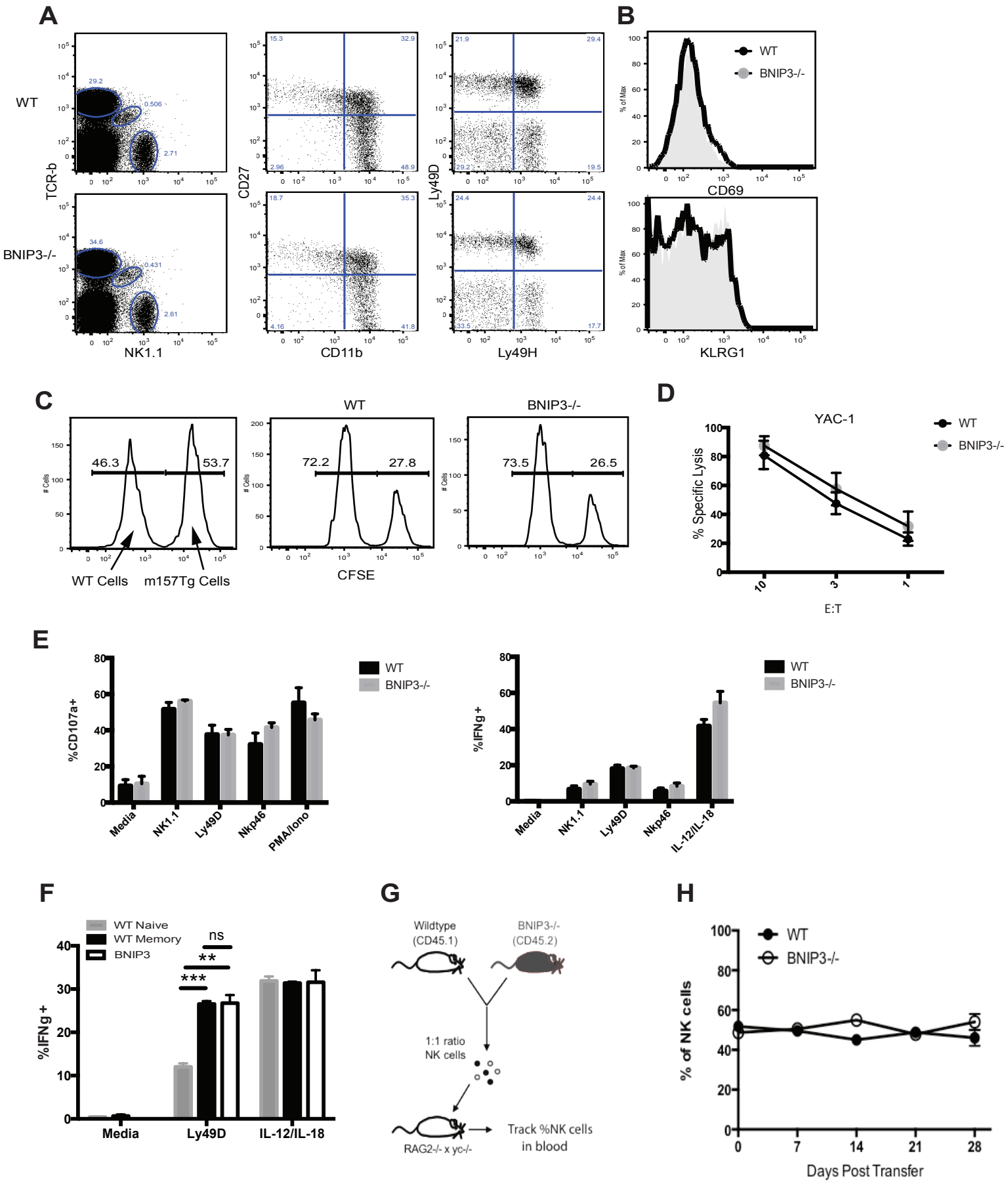

Figure S4, related to Figure 6. *Bnip3*$^{-/-}$ NK cells display normal development, functionality, and homeostasis. (A, **B**) Splenocytes from WT *or Bnip3*$^{-/-}$ mice were analyzed for the presence of mature NK cell markers by flow cytometry (**C**) Splenocytes from either WT or m157 transgenic mice were labeled with varying dilutions of CFSE and mixed at a 1:1 ratio and injected into either WT *or Bnip3*$^{-/-}$ recipients i.v. The percentage of each population is shown 48hrs after injection in harvested recipient spleens. (**D**) YAC-1 target cells were labeled with Cr^{51} and incubated with purified NK cells from WT *or Bnip3*$^{-/-}$ spleens for 5 hours at indicated effector to target ratios. Percent specific lysis is given as the percentage chromium release into cultured supernatant normalized to freeze thawed permeablized YAC-1 controls. (**E**) WT a*nd Bnip3*$^{-/-}$ NK cells were stimulated for 5 h in RPMI containing 5% FBS with recombinant mouse IL-12, IL-18, or with indicated plate-bound antibodies. CD107a and IFN-gamma intracellular staining was performed after incubation for 5 hours and analyzed by flow cytometry. (**F**) Quantitation of intracellular IFNγ staining of naïve or indicated adoptively transferred Ly49H^{+} NK cells at D28 PI after stimulation with plate-bound anti-Ly49D monoclonal antibodies or IL-12 and IL-18 for 5 h in RPMI containing 5% FBS. (**G**) NK cells f*rom B*nip3$^{-/-}$ (CD45.2) and WT (CD45.1) mice were mixed at a 1:1 ratio and adoptively transferred into RAG2$^{-/-}$ x γc$^{-/-}$ recipients. (**H**) Relative percentage of transferred WT *or Bnip3*$^{-/-}$ NK cells within the total NK cell populations in the blood at various time points PI.

Supplementary Figure 5

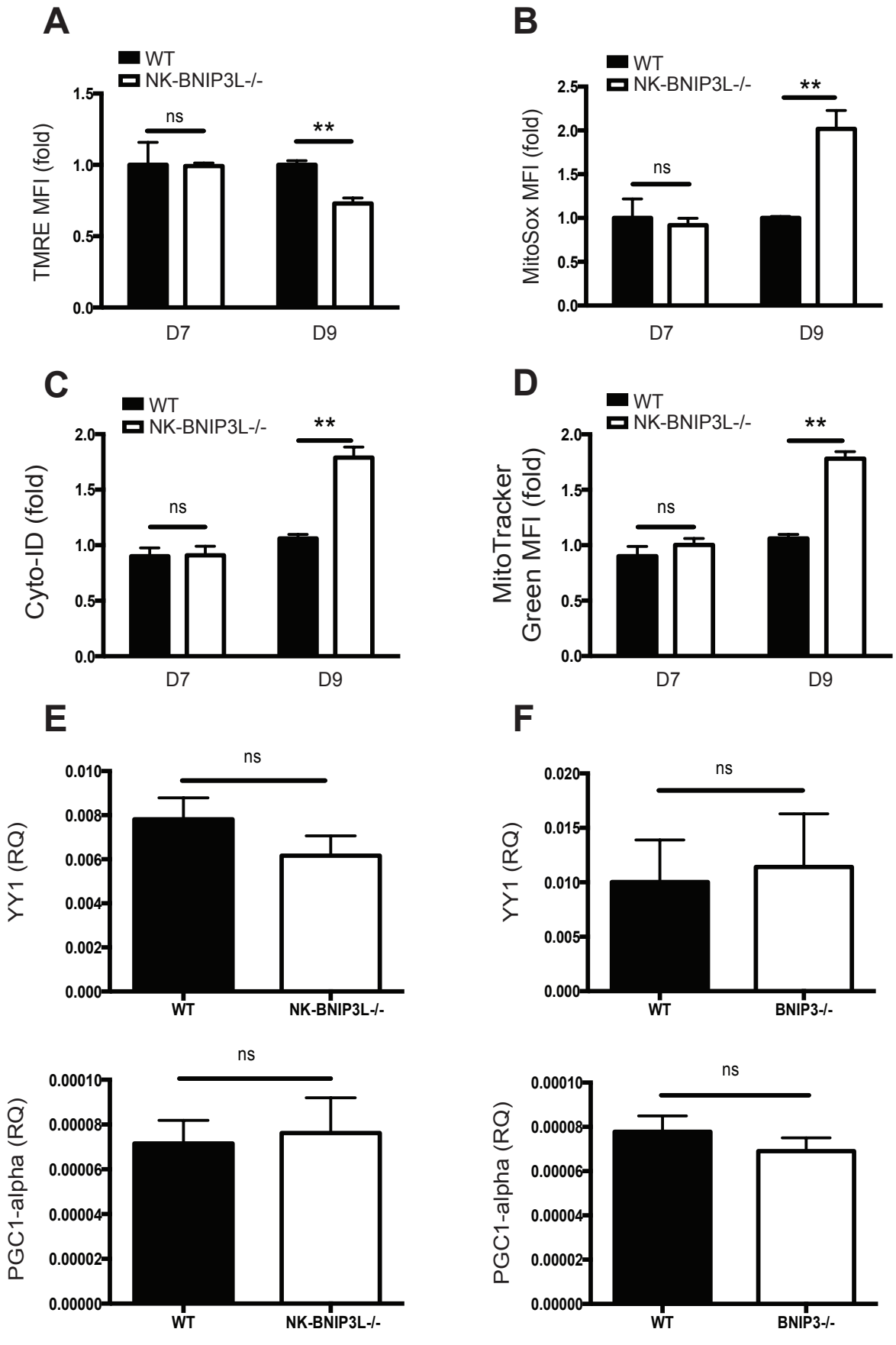

Figure S5, related to Figure 7. BNIP3 and BNIP3L are required for the removal of dysfunctional mitochondria, but not PGC1α or YY1 expression in NK cells following viral infection. (**A-F**) Equal numbers of WT *and NKp46^{iCre} x Bnip3l$^{fl/fl}$* (NK/NIP3L$^{-/-}$) NK cells were co-transferred into Ly49H-deficient mice, and infected with MCMV. Graphs show TMRE, MitoSox red, Cyto-ID, and MitoTracker Green MFI values for adoptively transferred *NK-Bnip3l$^{-/-}$* Ly49H^{+} cells (normalized to co-transferred WT controls in each recipient mouse and plotted as the fold change) at indicated time points PI. **(E,F**) Splenic Ly49H^{+} NK cells were sorted at (**E**) D7 a**n**d (**F**) D14 PI from recipient mice, and analyzed for PGC1α or YY1 mRNA by qRT-PCR compared to co-adoptively transferred WT controls (n=3 for each time point).