

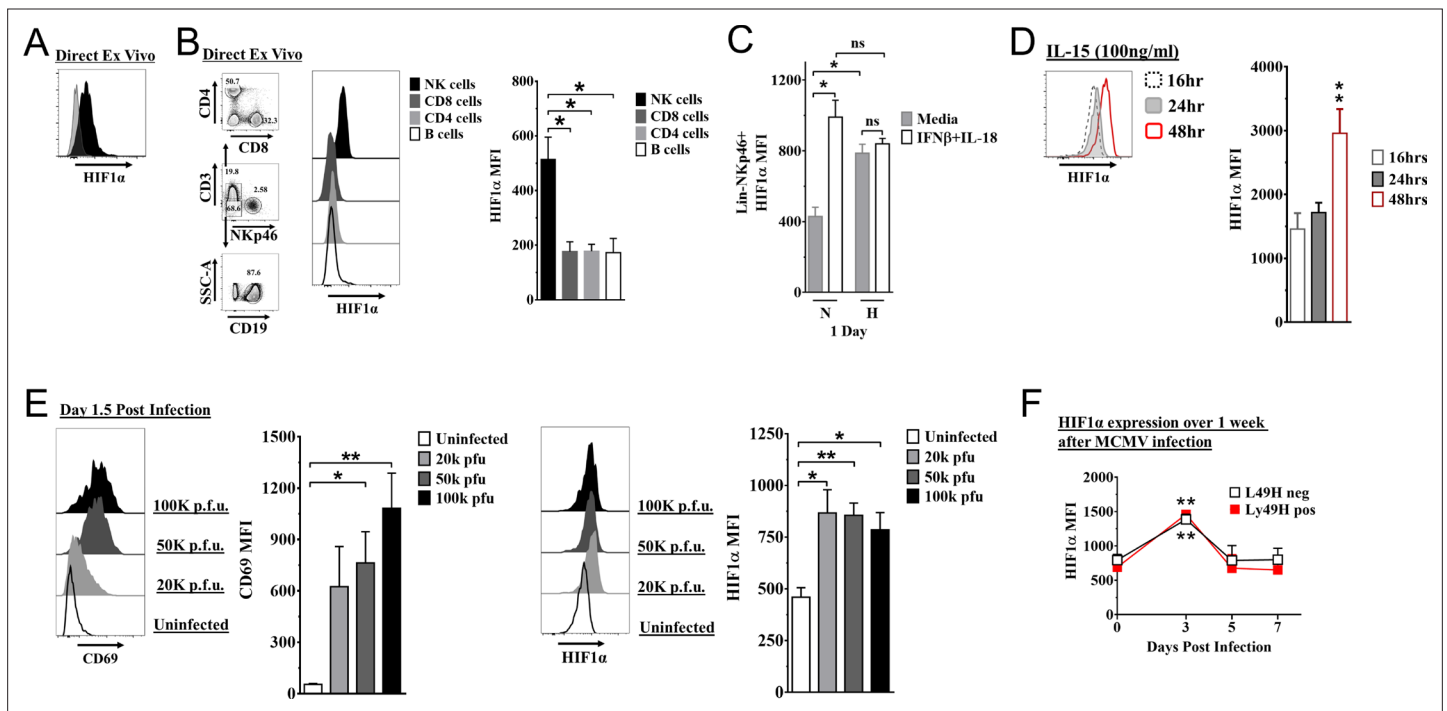


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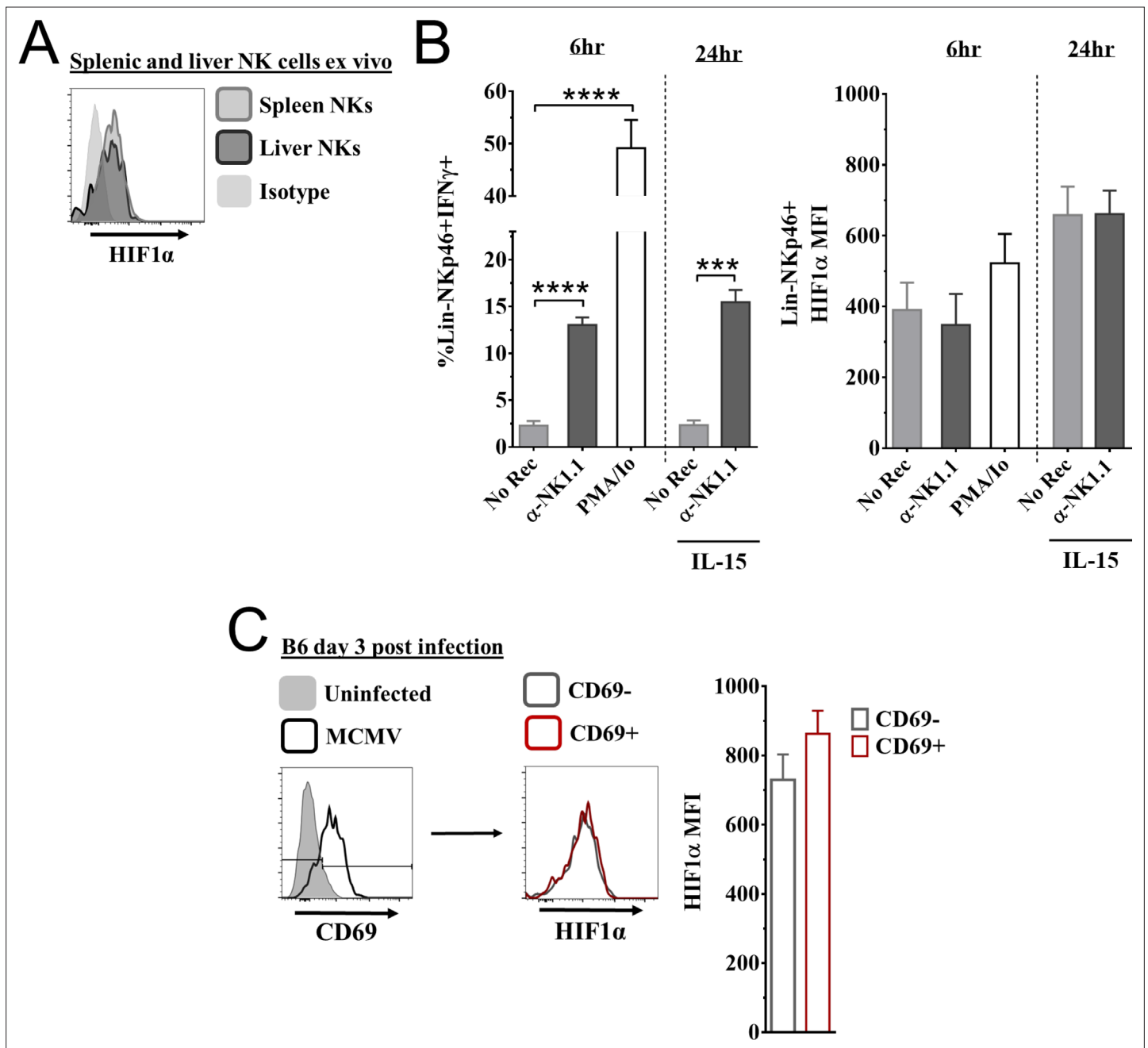
## Figures and figure supplements

HIF1 is required for NK cell metabolic adaptation during virus infection

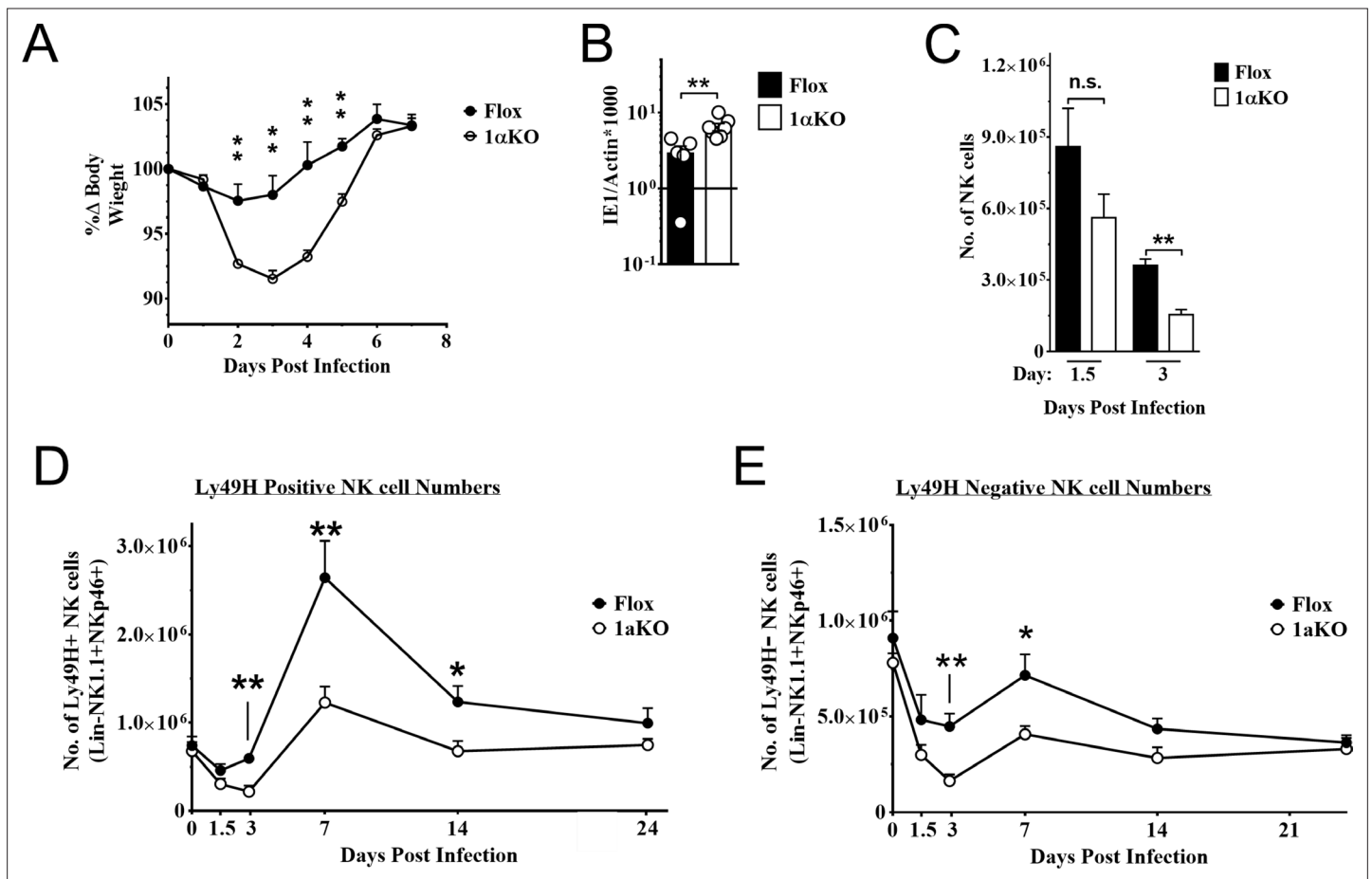
**Francisco Victorino et al**



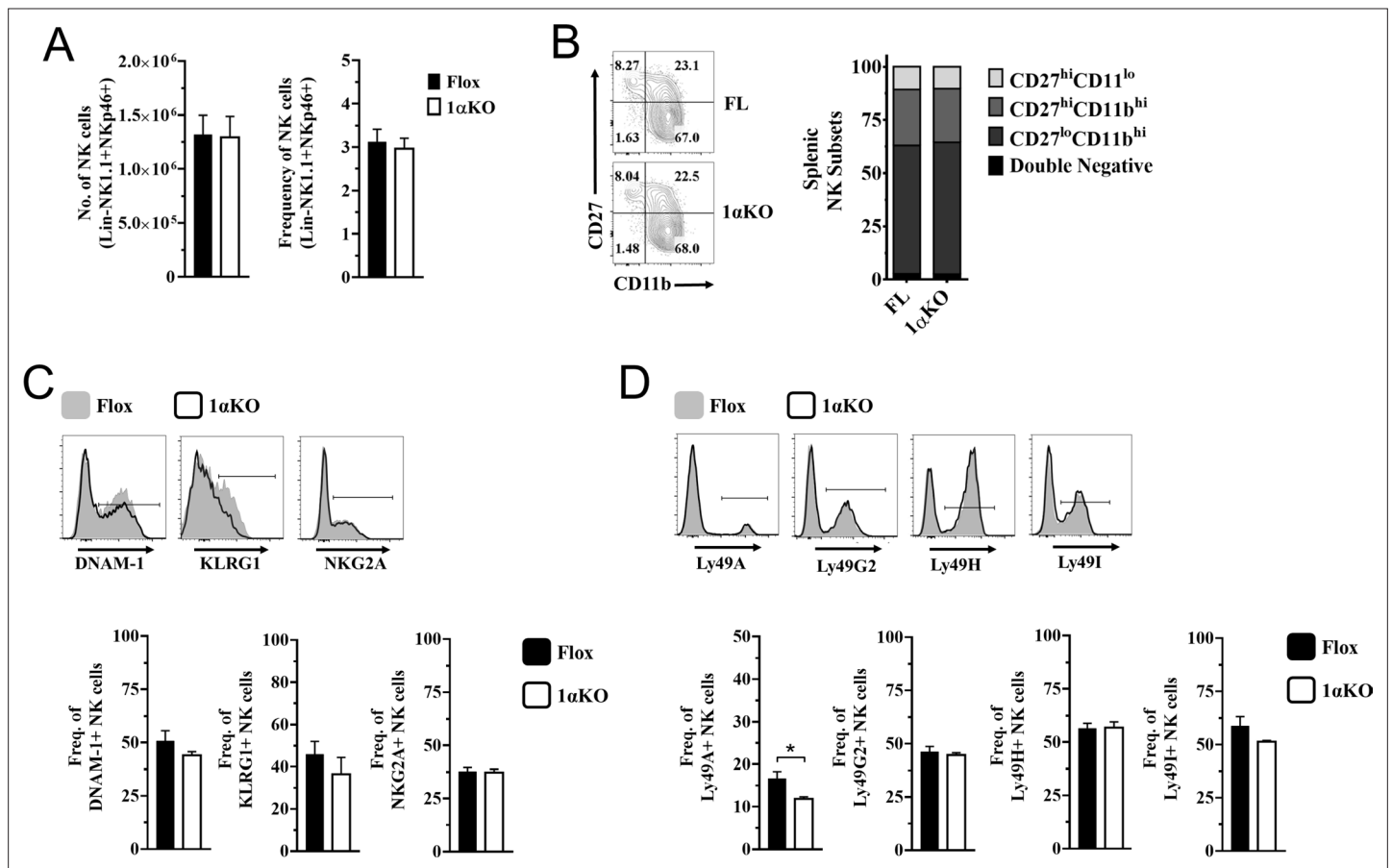
**Figure 1.** Hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) expression in natural killer (NK) cells. **(A)** HIF1 $\alpha$  protein expression in splenic NK cells (black) ex vivo compared to FMO control (gray) by flow cytometry. Representative experiment of two independent experiments. **(B)** Comparison of lymphocytes HIF1 $\alpha$  protein expression. Bar graphs show data from two independent experiments with four mice per group. **(C)** Spleens from C57BL/6 mice were prepared as in Materials and methods and were stimulated with IFN $\beta$  (200 units) + IL-18 (50  $\eta$  g/ml) for 24 hr in normoxia (N, 20%) or hypoxia (H, 1%). HIF1 $\alpha$  expression in NK cells was determined. Data are from two independent experiments with three mice per group. **(D)** Splenic NK cells were cultured in 25  $\eta$  g/ml IL-15 for 48 hr and HIF1 $\alpha$  expression was measured at 16, 24, and 48 hr. Data are from two independent experiments with four mice per group. **(E)** CD69 and HIF1 $\alpha$  was determined at day 1.5 post-infection (pi). Data are from 2–3 independent experiments with three mice per group. Statistical significance for uninfected versus indicated dose. **(F)** HIF1 $\alpha$  expression in Ly49H<sup>+</sup> or Ly49H<sup>-</sup> NK cells over 7 days from C57BL/6 mice that were infected with 50 K plaque forming unit (pfu) murine cytomegalovirus (MCMV). Statistical significance for d0 versus indicated day. Data are from two independent experiments with 3–7 mice per group. All data depict mean  $\pm$  SEM, with each data set containing data indicated number of mice per group from independent experiments. Unpaired t-test was performed (**A**, **B**, **D–F**) or one-way ANOVA (**C**). Statistical significance indicated by n.s., no significant difference; \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001; \*\*\*\* $p$ <0.0001.



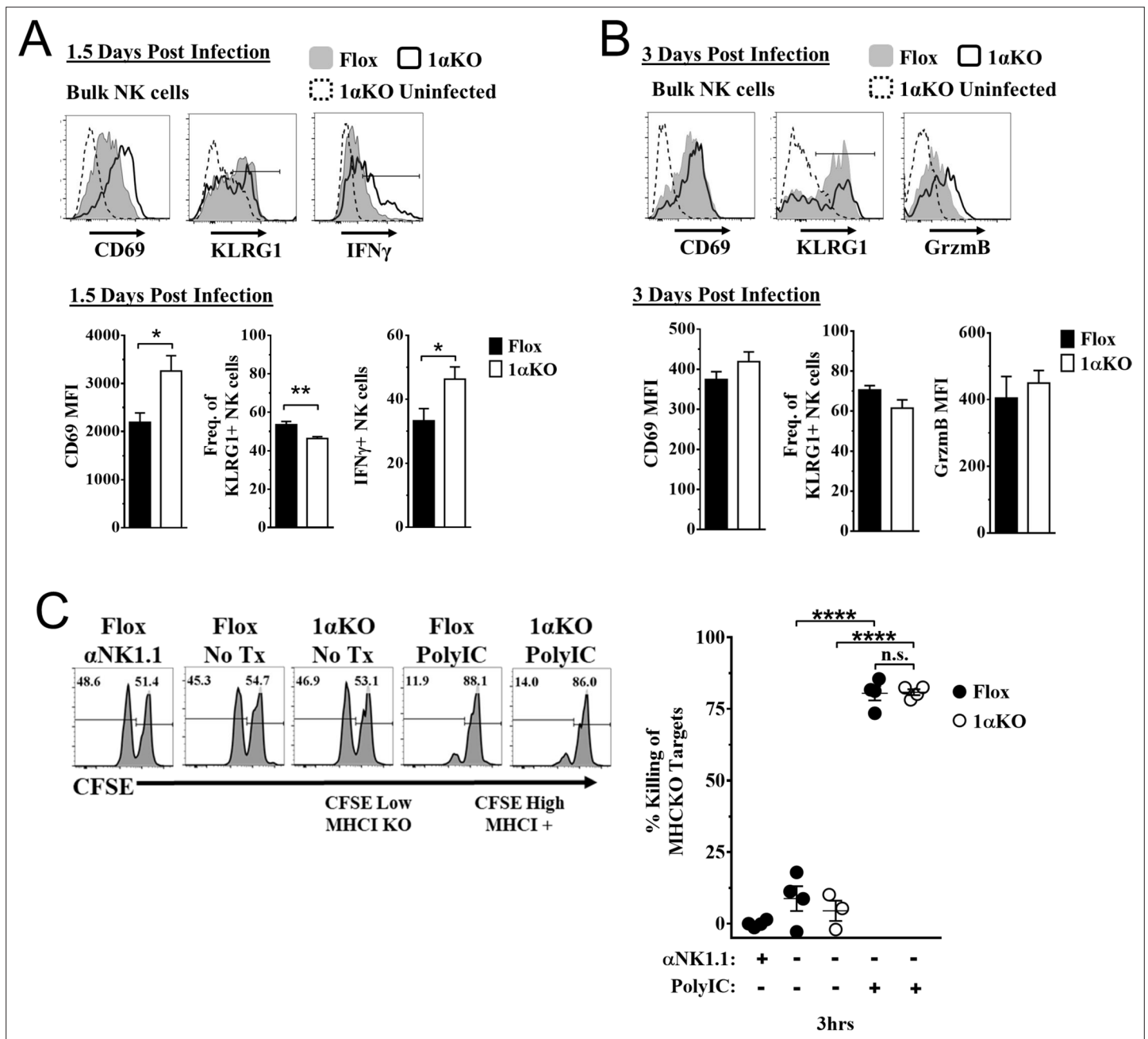
**Figure 1—figure supplement 1.** Expression of hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) in natural killer (NK) cells. **(A)** Representative histogram of HIF1 $\alpha$  expression in splenic and liver NK cells direct ex vivo. Data are from two independent experiments from five mice per group. **(B)** Splenocytes from C57BL/6 mice stimulated for 6 hr with either anti-NK1.1, PMA and ionomycin (PMA/Io), or no receptor stimulation (No Rec). For 24 hr culture, splenic NK cells were stimulated with anti-NK1.1, or no receptor stimulation (No Rec) in the presence of low IL-15. IFN  $\gamma$  (left) and HIF1 $\alpha$  (right) expression was measured in splenic NK cells. Data are from 2–3 independent experiments with 3–5 mice per group. **(C)** CD69 expression of splenic NK cells 3 days post murine cytomegalovirus (MCMV) infection, and HIF1 $\alpha$  expression in CD69 $^-$  and CD69 $^+$  NK cells (left) with quantification (right). Data are from three independent experiments with mice per group. Data depict mean  $\pm$  SEM, with each data set containing data indicated number of mice per group from independent experiments. Statistical significance indicated by n.s., no significant difference, \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001, \*\*\*\* $p$ <0.0001.



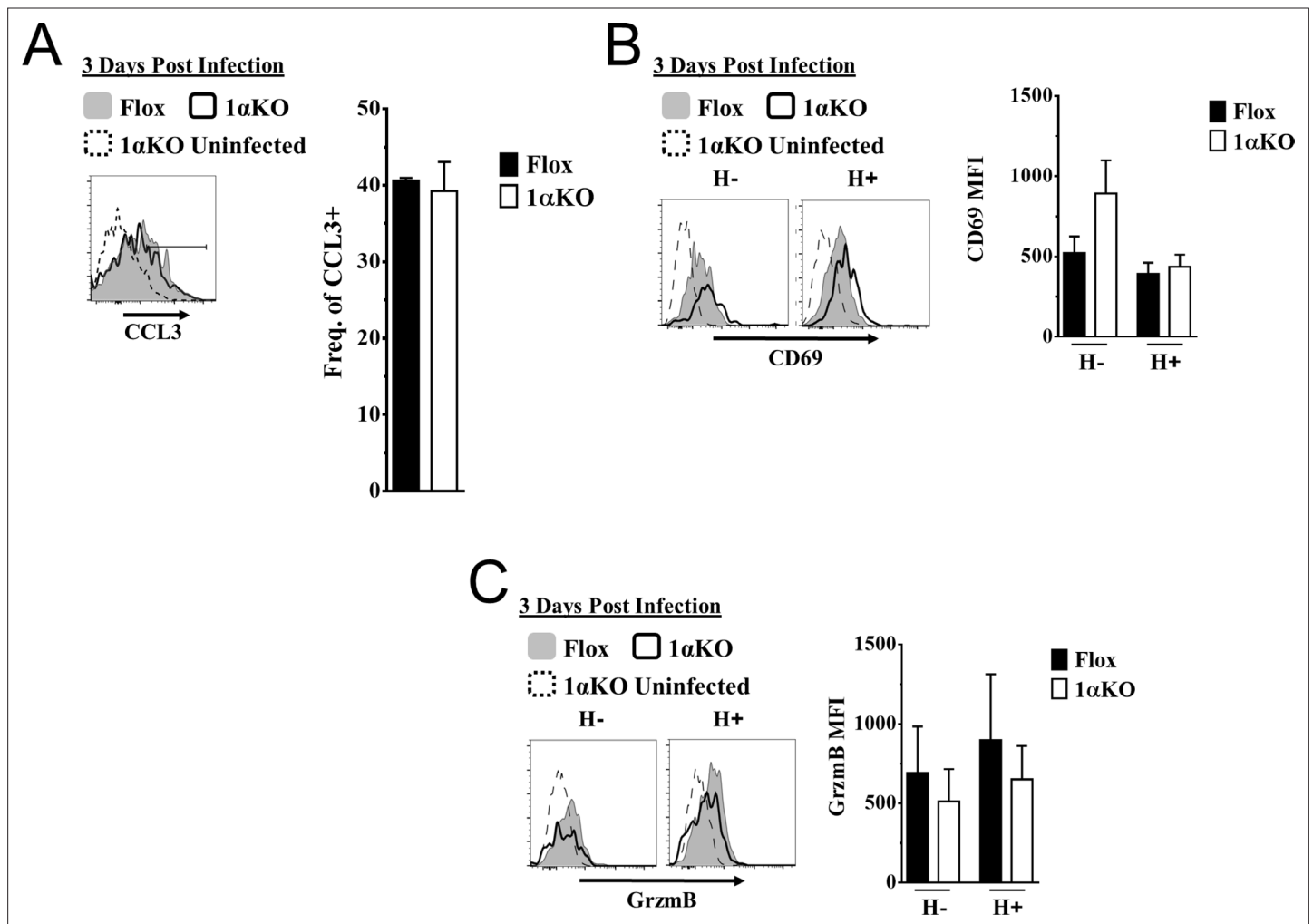
**Figure 2.** Natural killer (NK) cells require hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) for an optimal response to virus infection. **(A)** 1 $\alpha$ KO or FL control mice were infected with 50 K plaque forming unit (pfu) then monitored for weight loss over 7 days. Each data represents four mice from two independent experiments. **(B)** Splenic viral load in 1 $\alpha$ KO or FL control mice were infected with 50 K pfu then spleens harvested day 5 post-infection (pi). Data are pooled from four biologically independent experiments with a total of 5–7 mice in the indicated groups. **(C)** Number of bulk NK cells was quantified at days 1.5 and 3 pi for 1 $\alpha$ KO or FL control mice. Data are from 2–3 independent experiments with 4–6 mice per group. **(D)** 1 $\alpha$ KO or FL control mice were infected with 50 K pfu and Ly49H<sup>+</sup> NK cell expansion was determined at days 1.5, 3, 7, 14, and 24 pi. Data are from three independent experiments with 4–9 mice per group. **(E)** Quantification of 1 $\alpha$ KO or Flox Ly49H<sup>-</sup> NK cells at days 1.5, 3, 7, 14, and 24 post murine cytomegalovirus (MCMV) infection. Data are from 3–4 independent experiments with 3–4 mice per group. Data depict mean  $\pm$  SEM, with each data set containing data indicated number of mice per group from independent experiments. Unpaired t-test was performed on **(A–D)**. Statistical significance indicated by n.s., no significant difference; \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001; \*\*\*\* $p$ <0.0001.



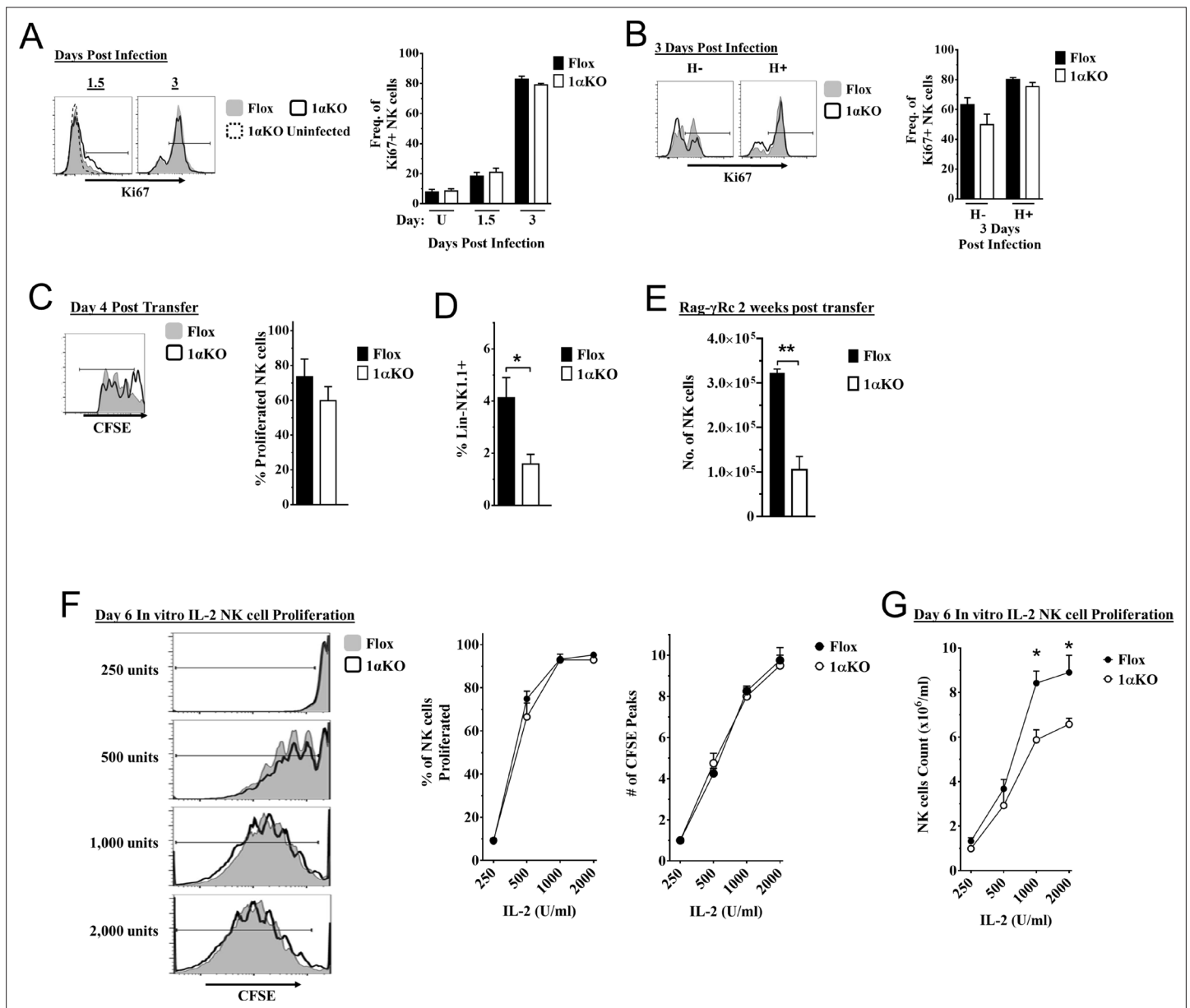
**Figure 2—figure supplement 1.** Normal development in hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) KO natural killer (NK) cells. **(A)** Quantification of NK cells absolute numbers (left) and frequencies (right) of NK cells in Flox controls and 1 $\alpha$ KO direct ex vivo. Data are from three independent experiments with 6–9 mice per group. **(B)** Representative contour plot (left) and quantification (right) of CD27 and CD11b maturation markers direct ex vivo in Flox controls and 1 $\alpha$ KO NK cells. Data are from two independent experiments with three mice per group. **(C)** Representative histograms (top) and frequencies (bottom) of DNAM-1, KLRG1, and NKG2A on NK cells of 1 $\alpha$ KO or FL control mice. Data are from 2–3 independent experiments with 3–7 mice per group. **(D)** Representative histograms (top) and frequencies (bottom) of Ly49A, Ly49G2, Ly49H, and Ly49I. Data are from 2–3 independent experiments with 3–7 mice per group. Data depict mean  $\pm$  SEM, with each data set containing data indicated number of mice per group from independent experiments. Statistical significance indicated by n.s., no significant difference, \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001, \*\*\*\* $p$ <0.0001.



**Figure 3.** Hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) is dispensable for natural killer (NK) cell effector function. **(A)** Representative histograms (top) and quantification (bottom) of CD69 MFI on NK cells and frequency of KLRG1<sup>+</sup> and IFN  $\gamma$  <sup>+</sup> NK cells at day 1.5 post-infection (pi) of 1 $\alpha$ KO or FL control mice. Data are from 2–3 independent experiments with 4–6 mice per group. **(B)** Representative histograms (top) and quantification (bottom) of CD69 and Granzyme B MFI (GrzmB) of NK cells, and frequency of KLRG1<sup>+</sup> NK cells at day 3 pi. Data are from two independent experiments with mice per group. **(C)** 1 $\alpha$ KO or FL control mice were injected with PBS or Poly (I:C) then 3 days later 20  $\times$  10<sup>6</sup> splenocytes at 1:1 ratio of CFSE-labeled MHC I-sufficient (CFSE<sup>High</sup>) and -deficient (CFSE<sup>Low</sup>) were intravenously transferred into mice. MHC-deficient splenocyte elimination was measured 3 hr post-transfer by flow cytometry. Data are from three independent experiments with 3–4 mice per group. Data depict mean  $\pm$  SEM, with each data set containing data indicated number of mice per group from independent experiments. Unpaired t-test was performed on **(A, B)** or one-way ANOVA **(C)**. Statistical significance indicated by n.s., no significant difference; \*p<0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001.

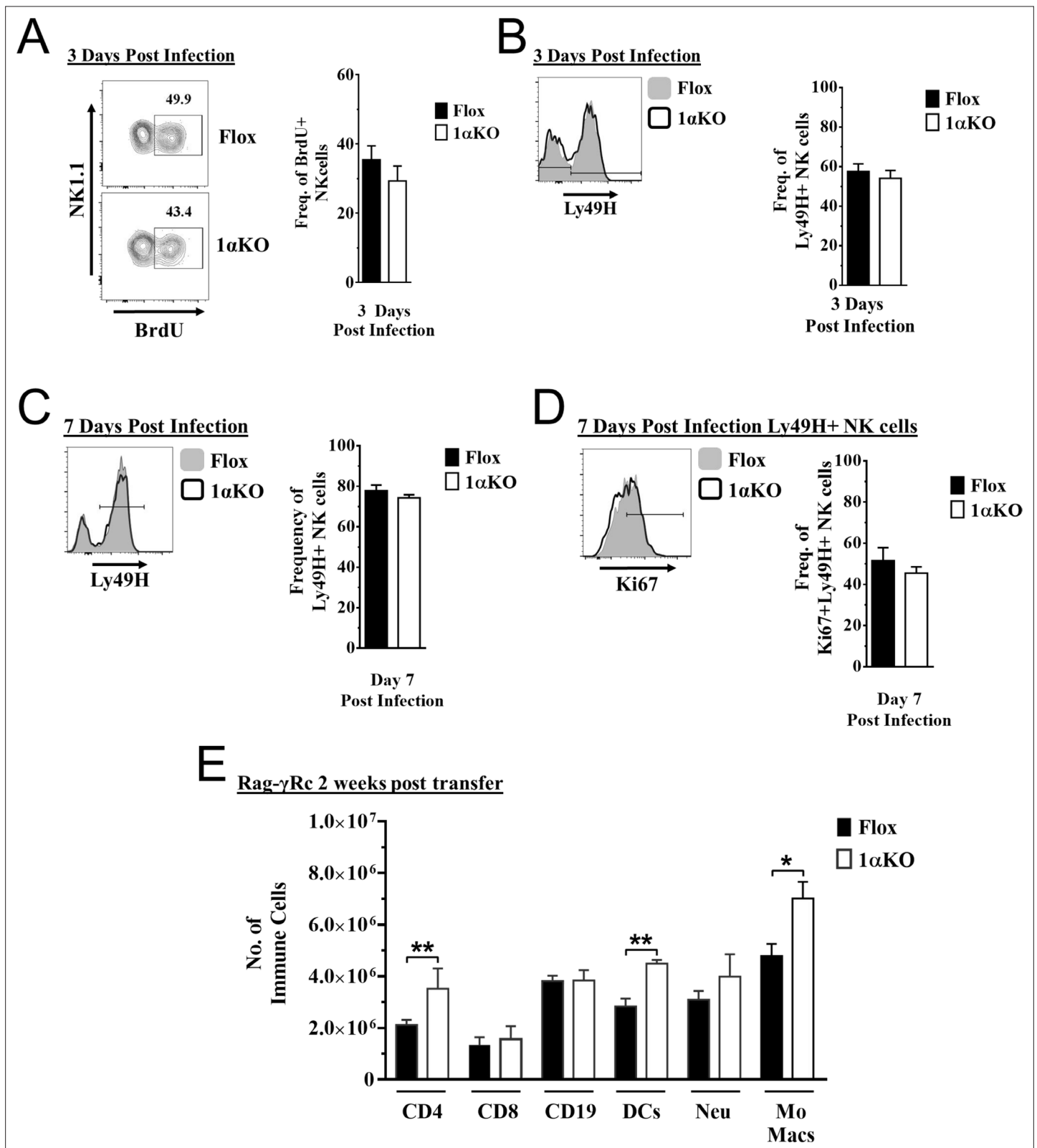


**Figure 3—figure supplement 1.** Ly49H<sup>+</sup> and Ly49H<sup>-</sup> hypoxia-inducible factor-1α (HIF1α) KO natural killer (NK) cells have similar activation compared to Flox controls. **(A–C)** 1αKO or Flox mice infected with murine cytomegalovirus (MCMV) received a dose of 50 K plaque forming unit (pfu) and analyzed as indicated. **(A)** Histogram (left) and quantification of frequency (right) represent CCL3 expression in NK cells at day 3 post MCMV infection. Data are from two independent experiments with mice per group. **(B)** Histograms (left) and quantification of (right) of CD69 MFI on Ly49H<sup>+</sup> and Ly49H<sup>-</sup> NK cells at day 3 post MCMV infection. Data are from two independent experiments with mice per group. **(C)** Representative histograms (left) and quantification of MFI (right) of Granzyme B (GrzmB) expression in Ly49H<sup>+</sup> and Ly49H<sup>-</sup> NK cells at day 3 post MCMV infection. Data are from two independent experiments with three mice per group. Data depict mean ± SEM, with each data set containing data indicated number of mice per group from independent experiments. Statistical significance indicated by n.s., no significant difference, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, \*\*\*\*p<0.0001.



**Figure 4.** Cell division in hypoxia-inducible factor-1α (HIF1α) KO natural killer (NK) cells is normal but numbers are reduced. (A, B) 1αKO or FL control mice infected with murine cytomegalovirus (MCMV) received a dose of 50 K plaque forming unit (pfu) and analyzed as indicated. (A) Ki67 expression with representative histograms (left) and frequency quantification (right) in 1αKO or FL control bulk NK cells at day 1.5 and day 3 post-infection (pi). Data are from three independent experiments with 5–7 mice per group. (B) Expression of Ki67 in Ly49H negative and positive NK cells shown in histogram (left) and frequencies quantified (right). Data are from two independent experiments with three mice per group. (C, D) Rag-γRc mice analyzed for CFSE + splenic NK cells from 1αKO or FL control mice 4 days post transfer, showing representative histogram (C, left) and quantification of total proliferation (C, right), and frequencies (D). Data are from two independent experiments with mice per group. (E) Rag-γRc mice analyzed on day 14 since post splenocyte transfer for numbers of NK cells in the spleen from 1αKO or FL control mice. Data are from two independent experiments with three mice per group. (F, G) In vitro proliferation assays were done using human recombinant IL-2 at different concentrations and time points indicated below. (F) Representative histograms (left), quantification of total proliferated cells (middle), and number of CFSE peaks (right) of CFSE labeled NK cells from 1αKO or FL control mice stimulated with different concentrations of IL-2 for 6 days. (G) Numbers of NK cells were counted from (F) and graphed in cells per 1 ml. Data are from two independent experiments with mice per group. Data depict mean ± SEM, with each data set containing data indicated number of mice per group from independent experiments. Unpaired t-test was performed on (A–G). Statistical significance indicated by n.s., no significant difference; \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001.

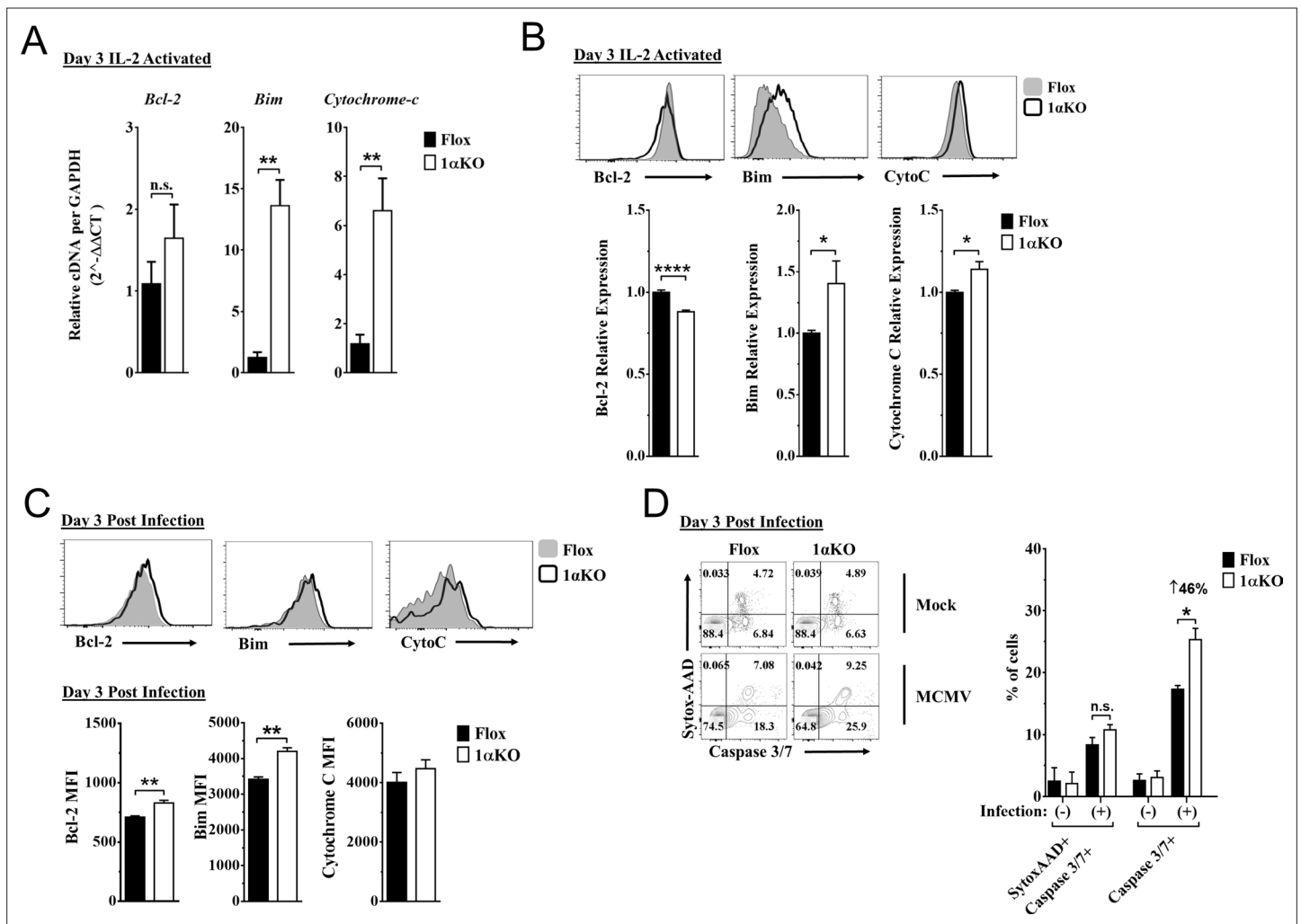




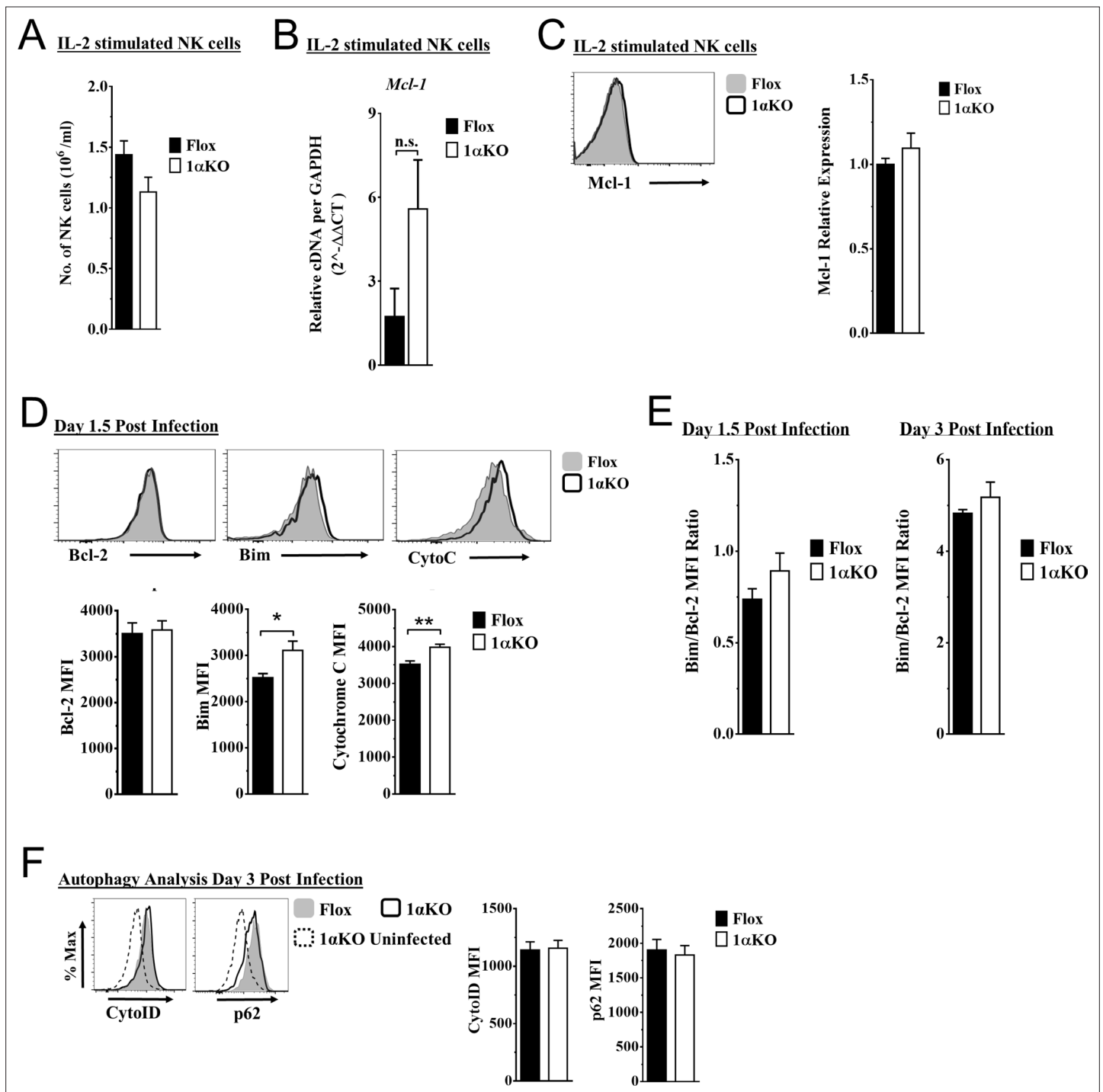
**Figure 4—figure supplement 1.** Proliferation is normal in hypoxia-inducible factor-1α (HIF1α) KO natural killer (NK) cells. (A–D) 1αKO or Flox mice infected with murine cytomegalovirus (MCMV) received a dose of 50 K plaque forming unit (pfu) and analyzed as indicated. (A) Representative contour plots (left) and quantification (right) of bulk splenic NK cells that were collected day 3 post MCMV infection and BrdU expression was determined. Data are from two independent experiments with mice per group. (B) Representative histogram (left) and quantification of frequency (right) of Ly49H+ NK cells. (C) Representative histogram (left) and quantification of frequency (right) of Ly49H+ NK cells. (D) Representative histogram (left) and quantification of frequency (right) of Ki67+ Ly49H+ NK cells. (E) Rag-γRc 2 weeks post transfer. Figure 4—figure supplement 1 continued on next page

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expressing NK cells at day 3 post MCMV infection. Data are from two independent experiments with mice per group. **(C)** Histogram (left) shows gating for Ly49H on NK cells at day 7 post MCMV infection and is quantified in the right graph. Data are from four independent experiments with mice per group. **(D)** Histogram that is representative of Ki67 expression in Ly49H + NK cells and quantification at day 7 post MCMV infection. Data are from four independent experiments with mice per group. **(E)** Rag- $\gamma$  Rc mice analyzed on day 14 since post splenocyte transfer for engraftment of lymphocytes and myeloid cells in the spleen. Data are from two independent experiments with three mice per group. Data depict mean  $\pm$  SEM, with each data set containing data indicated number of mice per group from independent experiments. Statistical significance indicated by n.s., no significant difference, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .



**Figure 5.** Hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) KO natural killer (NK) cells are predisposed to apoptosis. **(A)** mRNA transcripts for levels of apoptosis associated genes Bcl-2, Bim, and cytochrome c was measured in 1 $\alpha$ KO or FL control NK cells at day 3 post IL-2 stimulation. Data are from two independent experiments with mice per group. **(B)** Representative histograms (top) and quantification (bottom) of pro-survival relative protein expression of Bcl-2, Bim, and cytochrome c (Cyto-C) in NK cells 3 days post IL-2 stimulation. Data are from 2–5 independent experiments with 4–11 mice per group. **(C)** Representative histograms (top) and quantification (bottom) of Bcl-2 MFI, Bim MFI, and cytochrome c (Cyto-C) MFI in NK cells day 3 post murine cytomegalovirus (MCMV) infection. Data are from 2–3 independent experiments with 4–6 mice per group. **(D)** Representative contour plots (left) and frequency quantification (right) of necrotic cells (Caspase 3/7+ Sytox AAD+), and apoptotic cells (Caspase 3/7+ Sytox AAD-). Data are from two independent experiments with three mice per group. Data depict mean  $\pm$  SEM, with each data set containing data indicated number of mice per group from independent experiments. Unpaired t-test was performed on **(A–D)**. Statistical significance indicated by n.s., no significant difference; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .

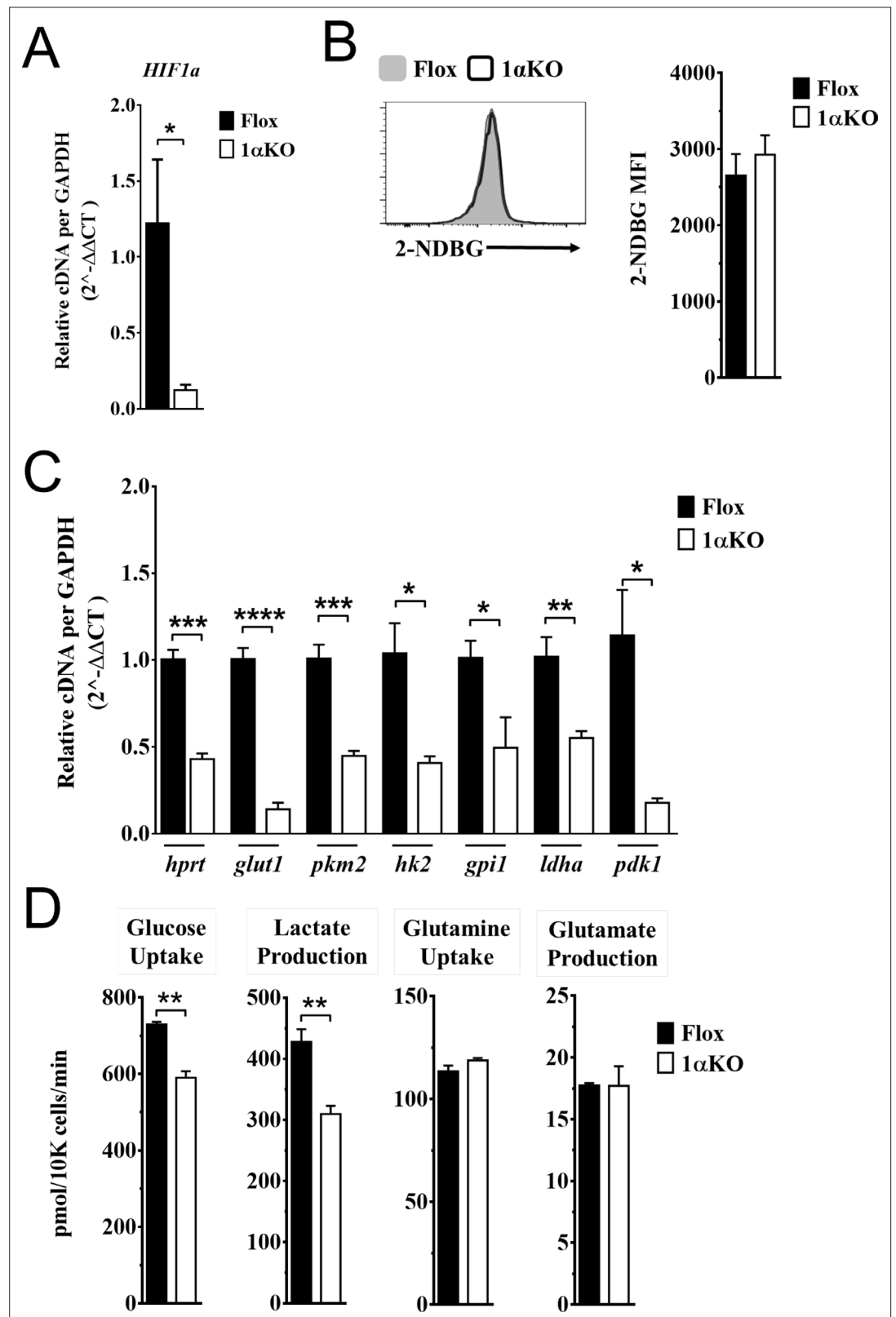


**Figure 5—figure supplement 1.** Hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) KO natural killer (NK) cells are predisposed to cell death. (A–C) Enriched NK cells stimulated with 2000 units/ml of IL-2 for 3 days then harvested and analyzed as indicated. Data represents mice per time point from two independent experiments. (A) Quantification of the number of NK cells in culture at day 3. Data are from two independent experiments with four mice per group. (B) mRNA levels for *Mcl-1* were quantified using qRT-PCR. Data are from two independent experiments with four mice per group. (C) Representative histogram (top) and quantification of relative protein expression pro-survival marker *Mcl-1*. Data are from two independent experiments with four mice per group. (D) Representative histograms (top) and quantification (bottom) of pro-survival protein Bcl-2 MFI, and pro-apoptotic proteins Bim MFI, and cytochrome c (Cyto-C) MFI in NK cells day 1.5 post murine cytomegalovirus (MCMV) infection. Data are from 2–3 independent experiments with 4–6 mice per group. (E) Ratio of Bim to Bcl-2 MFI quantified from Flox and 1 $\alpha$ KO NK cells at day 1.5 and day 3 post MCMV infection. Data are from three independent experiments with six mice per group for day 1.5 and two independent experiments with three mice per group for day 3. (F) Autophagy markers CytoID and p62 were measured day 3 post MCMV infection. Data are from two independent experiments with 4–5 mice per

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group. Data depict mean  $\pm$  SEM, with each data set containing data indicated number of mice per group from independent experiments. Statistical significance indicated by n.s., no significant difference, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

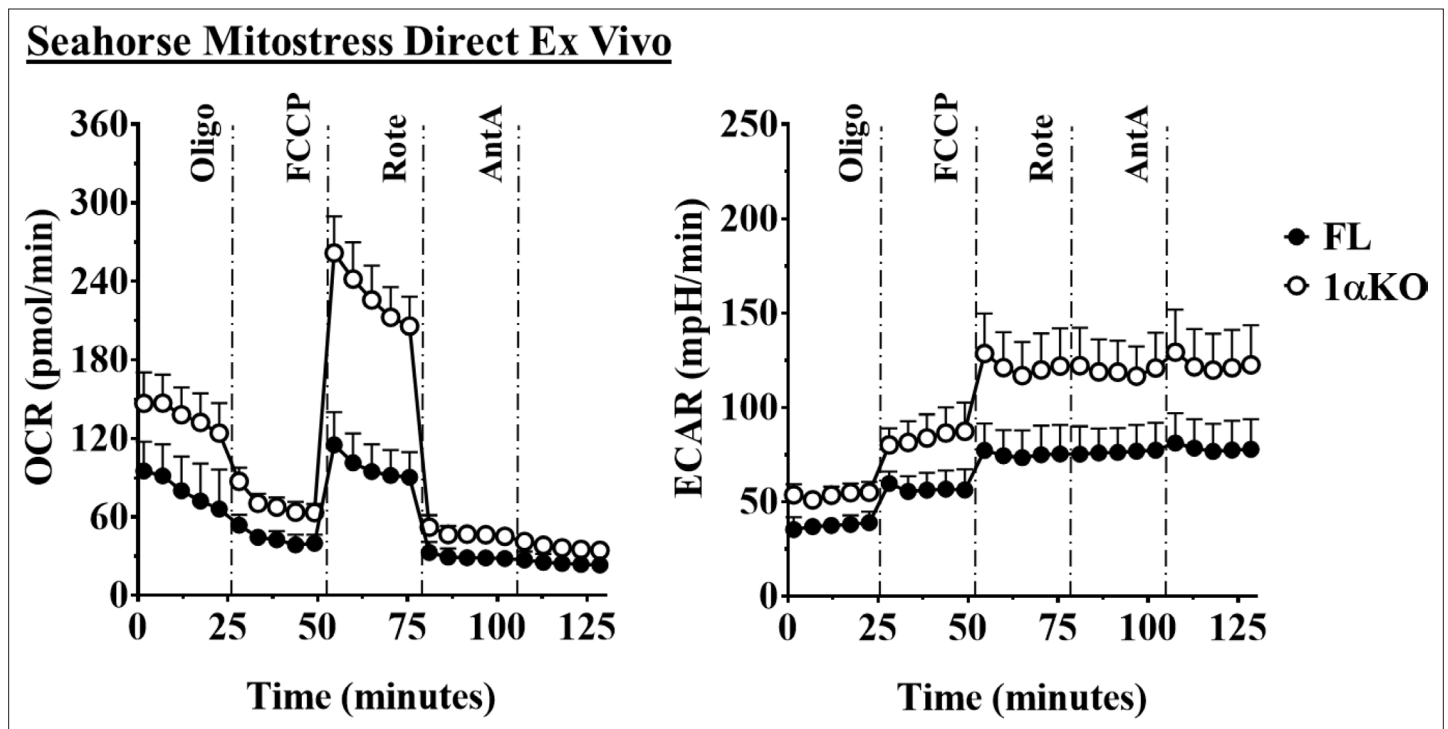


**Figure 6.** Impaired glycolytic activity contributes to impaired survival of hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) KO natural killer (NK) cells. Enriched NK cells from 1 $\alpha$ KO or FL control mice were IL-2 treated for 3 days then collected and analyzed as follows. **(A)** mRNA transcript levels of *HIF1 $\alpha$*  for both cohort of mice measured with qRT-PCR. Data are from two independent experiments with four mice per group. **(B)** Flow cytometry analysis of 2-NDBG MFI

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expression as a measure of glucose intake. Data are from three independent experiments with six mice per group. (C) mRNA transcripts levels for glycolysis genes *glut1*, *gpi1*, *hk2*, *hprt*, *ldha*, *pdk1*, and *pkm2* were measured with qRT-PCR. Data are from two independent experiments with four mice per group. (D) Media was collected from day 3 IL-2-activated NK cell cultures and using LC/MS, uptake of glucose (first panel) and glutamine (third panel), and production of lactate (second panel) and glutamate (fourth panel) was measured. Data are representative of two independent experiments with three mice per group. Data depict mean  $\pm$  SEM, with each data set containing data indicated number of mice per group from independent experiments. Unpaired t-test was performed on (A–D). Statistical significance indicated by n.s., no significant difference; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ ; \*\*\*\* $p < 0.0001$ .



**Figure 6—figure supplement 1.** Hypoxia-inducible factor-1 $\alpha$  (HIF1 $\alpha$ ) KO natural killer (NK) cells have dysregulated metabolism. Enriched NK cells from 1 $\alpha$ KO and Flox mice were subjected to a mitochondrial stress test, then, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured direct ex vivo. Data are from three independent experiments with 5–6 mice per group. Data depict mean  $\pm$  SEM, with each data set containing data indicated number of mice per group from independent experiments. Statistical significance indicated by n.s., no significant difference, \* $p$ <0.05, \*\* $p$ <0.01, \*\*\* $p$ <0.001, \*\*\*\* $p$ <0.0001.