Online Supplemental Material

Supplemental methods

Culture of human and murine neutrophils. Purified cells were resuspended at $5x10^6$/ml (human neutrophils) or $1x10^6$/ml (murine peripheral blood neutrophils) in RPMI with 10% fetal calf serum and 50 U/ml streptomycin and penicillin. Cells were cultured in the presence or absence of GEA3162 [30-100 μM], peptidoglycan [10 μg/ml], *E. coli* LPS [100 ng/ml], deferoxamine [300 μM], dimethyloxalylglycine [1-100 μM], heat killed SH1000 *S. aureus* [MOI 10:1] and serotype 2 *S. pneumoniae* strain D39 [MOI 10:1], in normoxia (19 kPa) or hypoxia (3 kPa). Hypoxia was attained using an *Invivo*$_2$ 400 hypoxic work station (Ruskinn Technology Ltd., Cardiff, UK) with a 1%O_2/5%CO_2/balance N_2 gas mix in the chamber, which resulted in an oxygen tension of 3kPa in culture media allowed to equilibrate overnight, as determined by automated blood gas analysis (NPT7, Radiometer Ltd., Crawley, UK).

RNA quantification. Primers were designed for human *HIF2A* (forward 5'CCAGACGTGCTGAGTCCGGC 3', reverse 5'GGCTTGCCATGCCTGACACCTT 3') and murine *Hif2a* (forward 5'TAAAGCGGCAGCTGGAGTAT 3', reverse 5'AGCTCCTGGAGGACCGTAGT 3') with cDNA subjected to PCR reactions using GoTaq® Flexi DNA Polymerase (Promega UK, Southampton). PCR products were run on 1.5% agarose gels, extracted using a QIAquick PCR purification kit (QIAGEN, Crawley, UK) and sequenced using BigDye® 3.1 sequencing kits on a 3730 DNA Analyser (Applied Biosystems, Foster, USA). Assays-on-demand gene expression TaqMan® MGB 6FAM dye-labelled products (Applied Biosystems) were used for murine *Hif1a*, *Hif2a*, *Actb* or human ACTB*,* *HIF1A*, *VEGF*, and *PAI-1* and assays performed according to manufacturer's instructions with 12.5 ng cDNA (murine) or 25 ng cDNA (human) per reaction. 6FAM dye-labelled probes and primers were designed for *PHD3* and *HIF2A* as previously reported [1]. Relative quantification for each gene was determined against *ACTB* expression.

Morpholino knockdown of *arnt-1*. The *arnt-1* morpholino (Genetools, Philomath, OR) was used as previously reported [2]. A standard control morpholino (Genetools) was used as a negative control.

Murine neutrophil function and receptor expression. To assess respiratory burst cells were cultured with 6 μM 2',7'-Dichlorofluorescin diacetate (Sigma-Aldrich) for 30 mins and then stimulated for a further 30 mins with heat killed serotype 2 *S. pneumoniae* (MOI 10:1) before FL1 geometric mean fluorescence was determined by flow cytometry. Cell surface receptor expression was determined by flow cytometry using anti-CD11b-PE or anti-L-selectin-PE antibodies (eBiosciences, Inc., Insight Biotechnology Ltd. Wembley, UK) following culture of cells in the presence or absence of LPS (10 ng/ml) for 1 hour. Murine neutrophil chemotaxis to KC (0-10 mM) over 1 hour was measured using Neuro Probe ChemoTx® microplates with a 5 μm filter (Neuro Probe Inc. Receptor Technologies, Ltd.). The number of cells in each well was expressed as a percentage of the positive control minus the percent migration of the chemokinesis control.

Cytokine analysis. Plasma and lavage supernatant cytokines were assayed using a multiplex cytokine kit (Meso Scale Discovery, Gaithersburg, Maryland, USA).

Apoptosis quantification in cells recovered from bronchoalveolar lavage. Apoptosis was assessed by flow cytometry with fluorescein isocyanate–labelled annexin V (BD Biosciences) and To-Pro®-3 (Molecular Probes®, Life Technologies) staining. Neutrophils were determined by forward/side scatter characteristics and Ly6G (eBiosciences) positivity.

Supplemental tables and figures

Supplemental table 1. *hif2a* primers used for PCR. Primers used to PCR amplify zebrafish *hif2a* homologues *a* and *b* and the dominant constructs. Dominant active primers are longer as site directed mutagenesis was performed to introduce each mutation individually in separate PCR reactions. PCR products for the wildtype *hif2a* variants were transformed into the pCR-II-TOPO vector (Invitrogen) and sequence verified. Each *hif2a* gene was then inserted into the pCS2+ vector (Invitrogen) from which site-directed mutagenesis was performed, followed by RNA transcription using SP6 enzyme and the mMessage-Machine kit (Ambion).

Supplemental figure 1. Expression of *HIF2A* and *HIF1A* mRNA is not altered by *S. aureus* or peptidoglycan. (A) Fold change in expression of *HIF2A* and *HIF1A* following culture with heat killed *S. aureus* (MOI 10:1) (hatched bars) or without (filled bars) for 3 or 5 hours. TaqMan® analysis of cDNA was performed with data normalized to *ACTB* expression. Data show mean and SEM fold change with respect to unstimulated samples at 3 hours, n=5. (B) Fold change in expression of *HIF2A* and *HIF1A* following culture with peptidoglycan (10 μg/ml) (shaded bars) or without (filled bars) for 3 or 5 hours. TaqMan® analysis of cDNA was performed with data normalized to *ACTB* expression. Data show fold change with respect to unstimulated samples at 3 hours, n=4.

Supplemental figure 2. Dominant active *hif2aa* delays resolution of neutrophilic inflammation. Dominant active (da) forms of *hif2a* RNA (177pg) were injected into the 1 cell stage zebrafish *mpx*:GFP embryos, tailfin transection performed at 2dpf, and neutrophils counted at 6 and 24hpi. Data shown are mean and SEM. (A) Injection of dominant active *hif2aa* variants did not alter the recruitment of neutrophils to the injury site after 6hpi when the tail was transected at 2dpf. Treatment with 100μM DMOG (filled bar) or DMSO vehicle control (open bars) was performed 2 hours before injury. n=14-17, performed as 2 independent experiments. (B) Dominant active *hif2aa* caused a significant increase in neutrophil number at 24hpi in the absence of DMOG treatment compared to phenol red injected negative controls. Treatment with 100μM DMOG (filled bar) or DMSO vehicle control (open bars) was performed at 4 hpi. Dominant active *hif2aa* alone was able to recapitulate the DMOG phenotype, whilst dominant active *hif2ab*

homologue did not. n=24, performed as 2 independent experiments. (C) Injection of dominant active *hif2a* variants led to a significant increase in total neutrophil numbers at 2dpf if they were co-injected. n=14-17, performed as 2 independent experiments. (D) 24hpi neutrophil counts in the *hif2aa+b* overexpressing fish at 2dpf following co-injection with control (open bars) and *arnt-1* morpholinos (filled bars). n=8 performed as 2 independent experiments. (E) *hif2aa* G487R or G487W RNA (177pg) or dominant active (da) *hif1ab* control was injected into 1 cell stage zebrafish *mpx*:GFP embryos. Tailfins were transected 2 dpf. Neutrophils were counted at 24, 48 and 72 hpi.

Supplemental figure 3. Myeloid-specific HIF-2α deficiency does not affect *in vitro* apoptosis. (A) Neutrophils from mice with myeloid-specific deletion of *Hif2a* (filled bars) or littermate controls (open bars) were cultured for 5 or 10 hours in normoxia or hypoxia before apoptosis was assessed by morphology. Data are mean and SEM for n=3. (B) Representative cytospin images of HIF-2α deficient neutrophils cultured in normoxia or hypoxia for 9 hours. Arrowheads indicate apoptotic neutrophils.

Supplemental figure 4. Murine neutrophils deficient in HIF-2α have normal function and preserved receptor expression. Functional assays. (A) Respiratory burst: neutrophils from C57BL/6 (open bars), *Hif1a*$^{flox/flox}$;*LysMcre*$^{+/-}$ (hatched bars) and *Hif2a*$^{flox/flox}$;*LysMcre*$^{+/-}$ (filled bars) mice were pre-incubated with 6 μM DCF (30 mins) and stimulated with heat-inactivated *S. pneumoniae* (MOI 10:1) and FL1 geometric mean fluorescence determined by flow cytometry (n=3). (B-C) Receptor expression: neutrophils from C57BL/6 (open bars), *Hif1a*$^{flox/flox}$;*LysMcre*$^{+/-}$ (hatched bars) and *Hif2a*$^{flox/flox}$;*LysMcre*$^{+/-}$ (filled bars) mice were cultured in the presence/absence of LPS (10 ng/ml), stained with PE-anti-CD11b (B) or PE-anti-L-selectin (C) and geometric mean fluorescence determined by flow cytometry (n=3). (D) Phagocytic index was calculated on cytospins of neutrophils from C57BL/6 (open bars), *Hif1a*$^{flox/flox}$;*LysMcre*$^{+/-}$ (hatched bars) and *Hif2a*$^{flox/flox}$;*LysMcre*$^{+/-}$ (filled bars) mice that were stimulated with opsonised zymosan A (0.2 mg/ml) for 30 minutes. Data are mean and SEM (n=3). (E) Chemotaxis: neutrophil migration to KC (0-10 μM) across a 5 μm filter was assessed in neutrophils from *Hif2a*$^{flox/flox}$;*LysMcre*$^{-/-}$ (open bars) and *Hif2a*$^{flox/flox}$;*LysMcre*$^{+/-}$ (filled bars) mice. Data are mean and SEM (n=3).

Supplemental figure 5. Mice with myeloid-specific deficiency of HIF-2α have preserved cytokine production following LPS-induced acute lung injury. (A-B) *Hif2a*$^{flox/flox}$;*LysMcre*$^{+/-}$ mice and *Hif2a*$^{flox/flox}$;*LysMcre*$^{-/-}$ controls have normal baseline bronchoalveolar lavage cell counts (A) and differential counts (B). (C-J) *Hif2a*$^{flox/flox}$;*LysMcre*$^{+/-}$ mice and littermate *Hif2a*$^{flox/flox}$;*LysMcre*$^{-/-}$ controls were challenged with nebulised LPS (3 mg). Bronchoalveolar lavage was performed at 6, 24, 48, 72 and 120 hours. Cytokines in plasma and bronchoalveolar lavage fluid were assessed using a multiplex cytokine kit. Levels of (C) IL-10, (D) KC, (E) IL-6, (F) IL-12 and (G) TNF-α are shown for control (open bars) and HIF-2α deficient mice (filled bars) as mean ± SEM for a minimum n=4. Cytokine levels at 72 and 120 hours were below the limits of detection of the assays. (H) Efferocytosis rates were determined from cytospins of lavaged cells recovered 48 hours post LPS challenge. (I-J) Rates of neutrophil apoptosis were determined in lavaged cells recovered 120 hours post LPS by (I) morphology or (J) flow cytometry analysis of annexinV/To-Pro-3 staining.

Supplemental figure 6. Preserved expression of apoptotic regulators in HIF-2α deficient inflammatory neutrophils. *Hif2a*$^{flox/flox}$;*LysMcre*$^{+/-}$ mice and littermate *Hif2a*$^{flox/flox}$;*LysMcre*$^{-/-}$ controls were challenged with nebulised LPS (3 mg). Bronchoalveolar lavage was performed 6 or 24 hours after the challenge. RNA was extracted from BAL cells and real time PCR was performed for (A) *Bclxl,* (B) *Siva1,* (C) *Mcl1*, or (D) *Nfkb*. Expression was normalised to beta actin. Data are mean and SEM for minimum n=3.

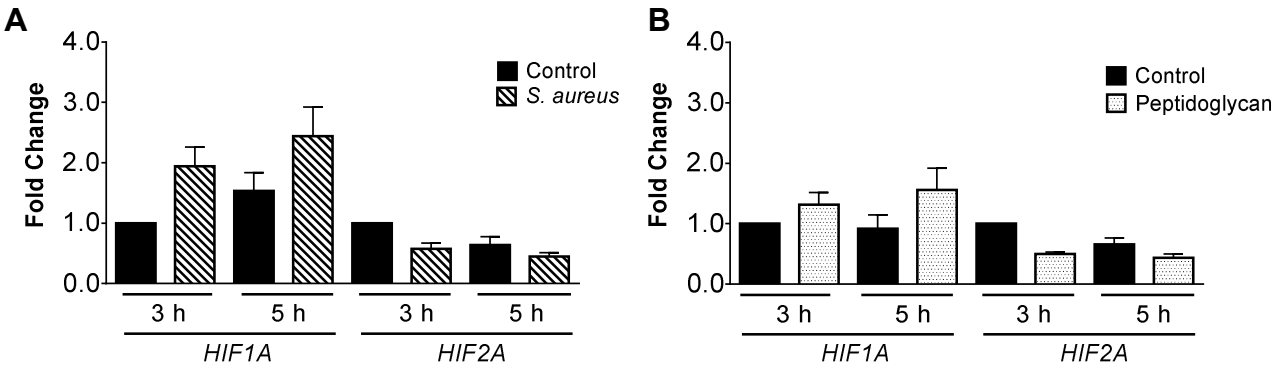
Supplemental figure 7. Genotyping of cells recovered from bronchoalveolar lavage fluid of mice following bone marrow transfer. C57BL/6 mice were irradiated and injected with bone marrow cells from *Hif2a*$^{flox/flox}$;*LysMcre*$^{+/-}$ mice (KO→C57) or littermate *Hif2a*$^{flox/flox}$;*LysMcre*$^{-/-}$ animals (WT→C57). After 5 weeks these mice were challenged with nebulised LPS (3 mg). Bronchoalveolar lavage was performed 6 or 48 hours after the challenge. DNA was extracted from BAL cells and amplified using primers for floxed or wild-type *Hif2a* alleles.[3] Control lanes include C57BL/6 wild-type DNA (C57) and *Hif2a*$^{flox/flox}$;*LysMcre*$^{+/-}$ DNA (+ve) obtained from ear-clips. Representative images of n=10 per group. Agarose gel images were inverted using Adobe Photoshop.

Supplemental references

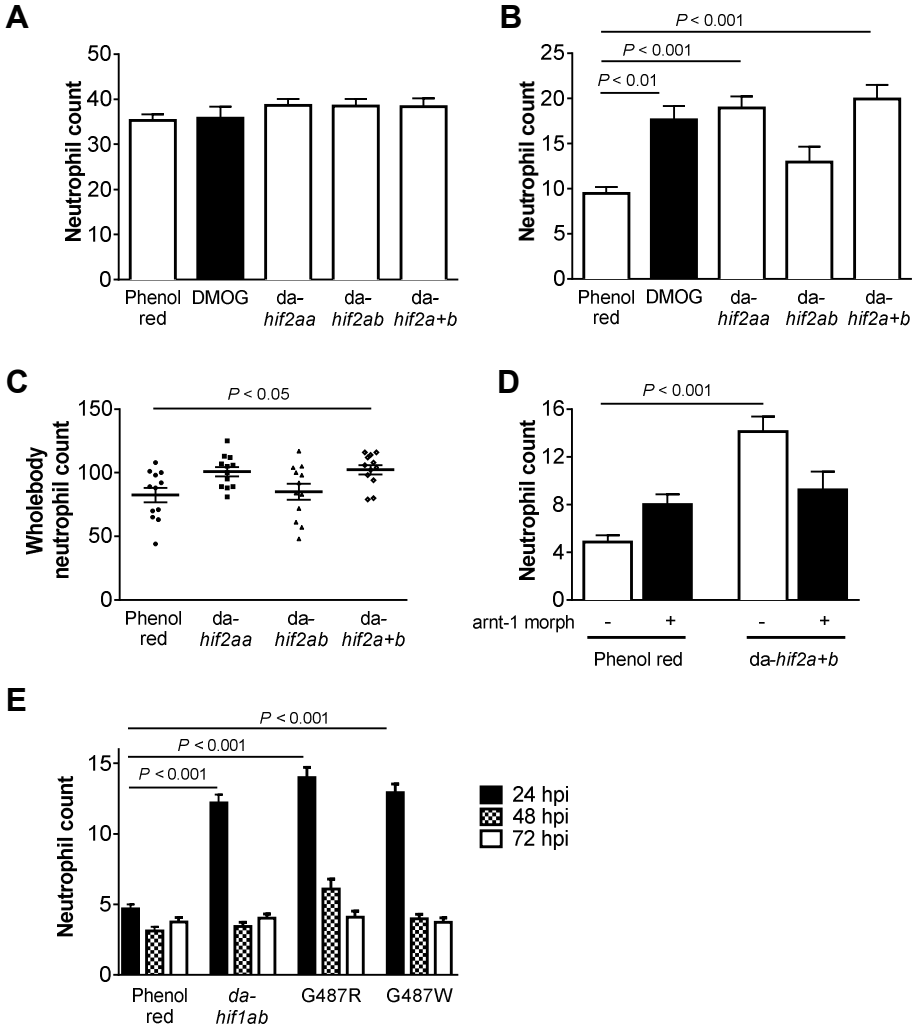
1. Walmsley SR, Chilvers ER, Thompson AA, et al. Prolyl hydroxylase 3 (PHD3) is essential for hypoxic regulation of neutrophilic inflammation in humans and mice. *J Clin Invest*. 2011;121(3):1053-1063.
2. Prasch AL, Tanguay RL, Mehta V, Heideman W, Peterson RE. Identification of zebrafish ARNT1 homologs: 2,3,7,8-tetrachlorodibenzo-p-dioxin toxicity in the developing zebrafish requires ARNT1. *Mol Pharmacol*. 2006;69(3):776-787.
3. Gruber M, Hu CJ, Johnson RS, Brown EJ, Keith B, Simon MC. Acute postnatal ablation of Hif-2alpha results in anemia. *Proc Natl Acad Sci U S A*. 2007;104(7):2301-2306.

Supplemental table 1.

PCR Product	Forward Primer	Reverse Primer
hif2aa	5'-CACACCTGGACAAAGCCTCT-3'	5'-CTGATTGCTCACCCCTGTTT-3'
hif2ab	5'-AGAGCGGCGTAAGGAGAAAT-3'	5'-GGATGAAGAGGGTGAATGGA-3'
hif2aa Δ330	5'- TCATGAGACTGGCTATCAGC -3'	5'-ATCGATTCAGGAGTTGCGGCTGTTGTA-3'
hif2ab Δ330	5'- TCATGAGACTGGCTATCAGC -3'	5'- ATCGATTCAGGAGTTGCGGCTGTTGTA -3'
hif2aa P347A	5'-TAGCGCAGTTAGCGGCTATGCCAGGAGAC-3'	5'-GTCTCCTGGCATAGCCGCTAACTGCGCTA-3'
hif2aa P481G	5'-CCTGGAGACTCTCGCTGGATACATCCCAATGGAC-3'	5'-GTCCATTGGGATGTATCCAGCGAGAGTCTCCAGG-3'
hif2aa N753A	5'-GCGATATGACTGTGAGGTAGCCATGCCTCTACAAGGAAAC-3'	5'-GTTTCCTTGTAGAGGCATGGCTACCTCACAGTCATATCGC-3'
hif2ab P343A	5'-ACGCAGCTGGCAGCTACACCTGGGG-3'	5'-CCCCAGGTGTAGCTGCCAGCTGCGT-3'
hif2ab P469G	5'-ACTTGGAGACACTGGCTGGCTATATCCCCATGGATG-3'	5'-CATCCATGGGGATATAGCCAGCCAGTGTCTCCAAGT-3'
hif2ab N755A	5'-TTACGACTGCGAGGTCGCTGTCCCGTTGCAAGGC-3'	5'-GCCTTGCAACGGGACAGCGACCTCGCAGTCGTAA-3'
hif2aa G487R	5'-ACATCCCAATGGACCGCGAGGACTTCCAG-3'	5'-CTGGAAGTCCTCGCGGTCCATTGGGATGT-3'
hif2aa G487W	5'-ATACATCCCAATGGACTGGGAGGACTTCCAGCTGC-3'	5'-GCAGCTGGAAGTCCTCCCAGTCCATTGGGATGTAT-3'



Supplemental figure 1



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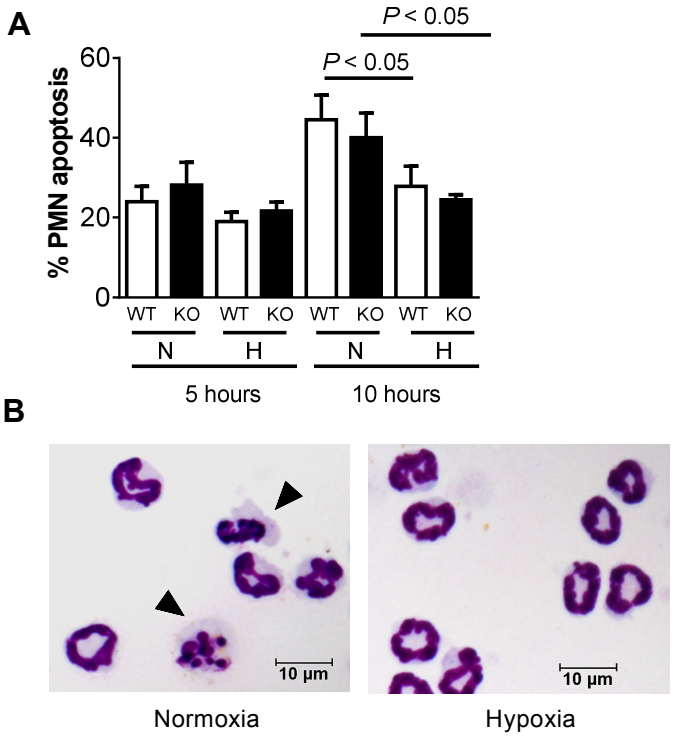

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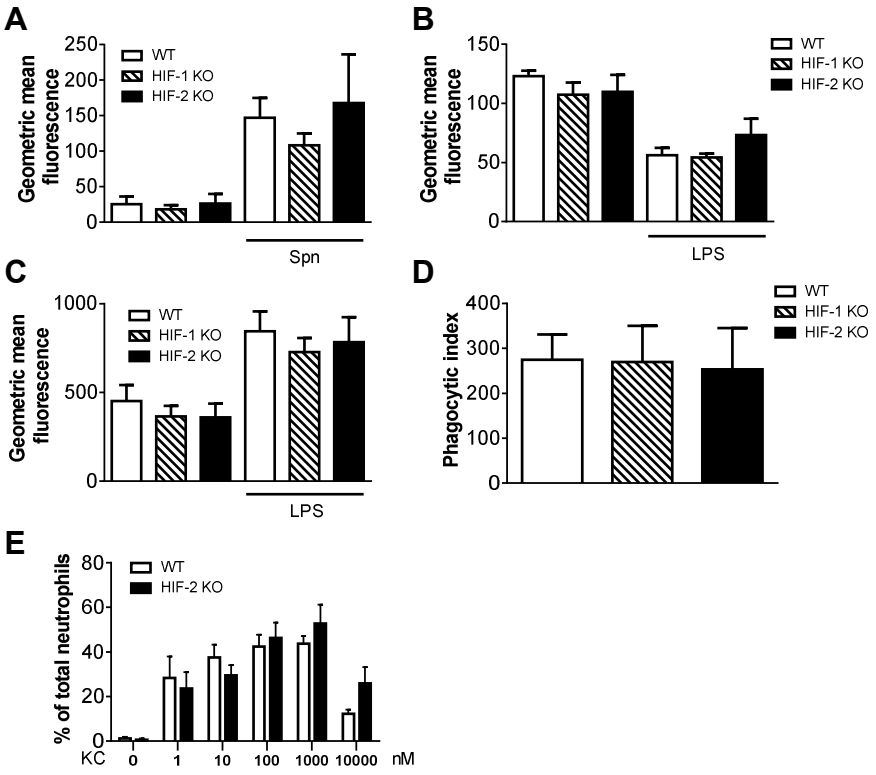

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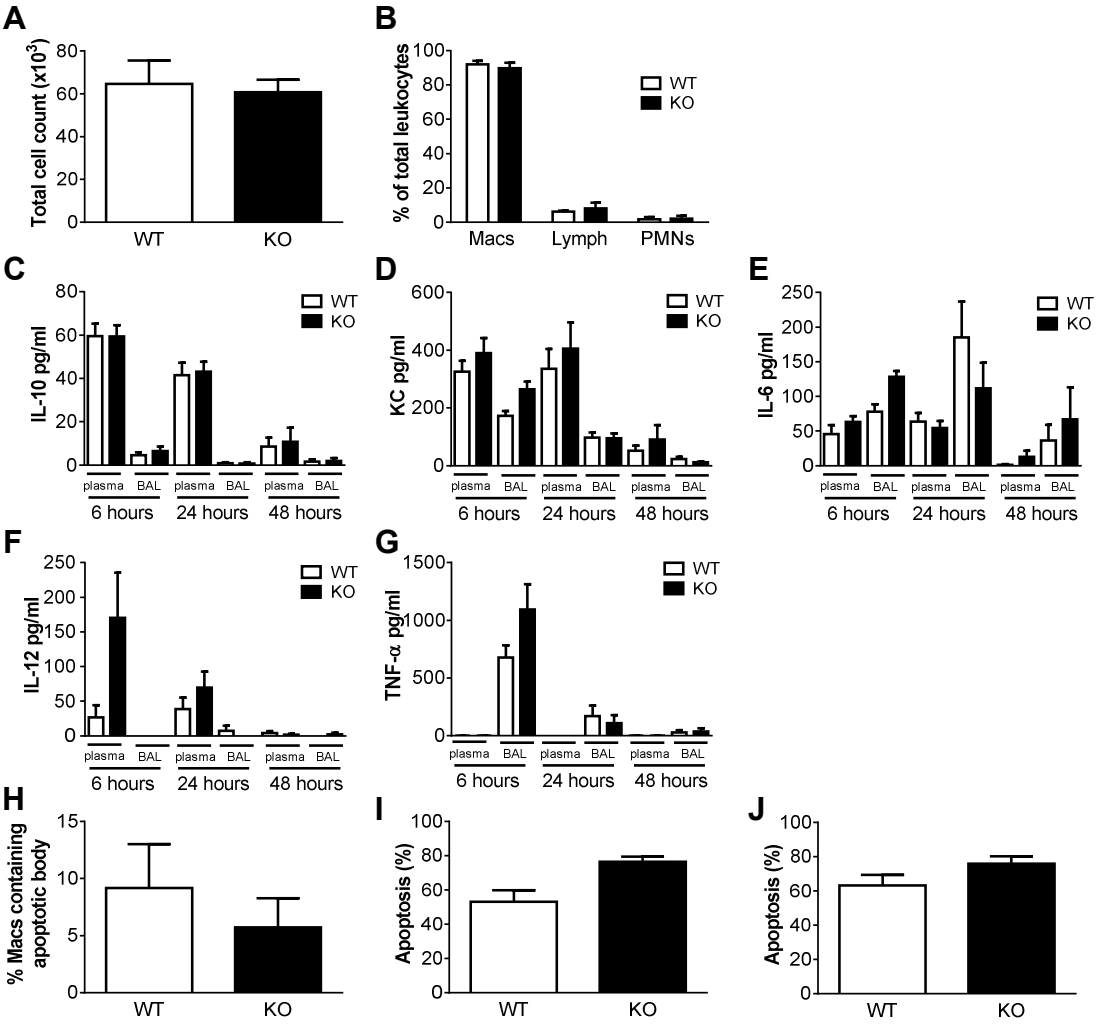

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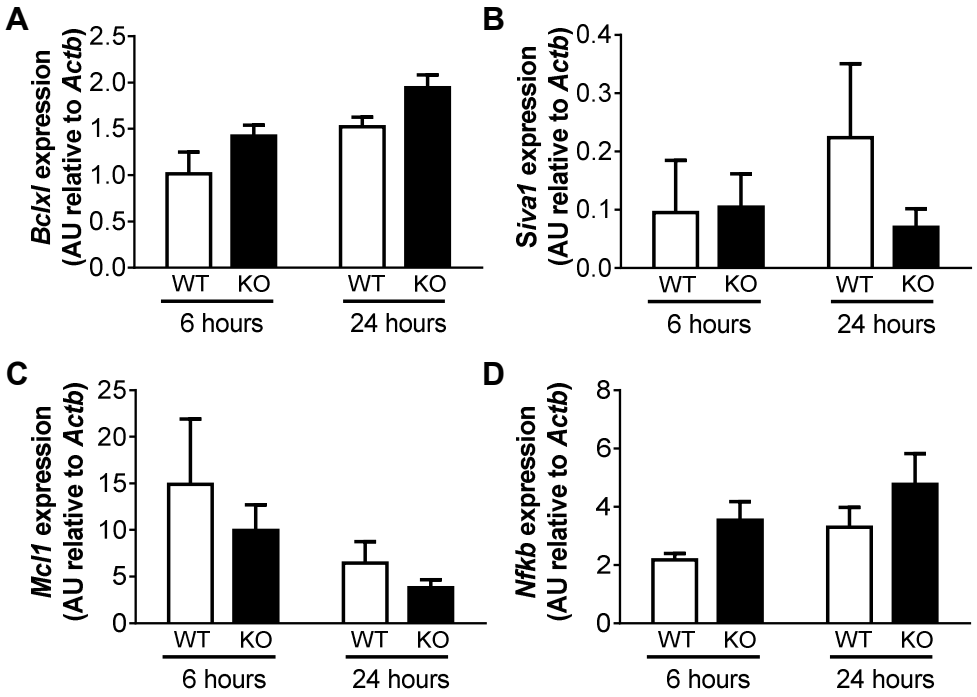

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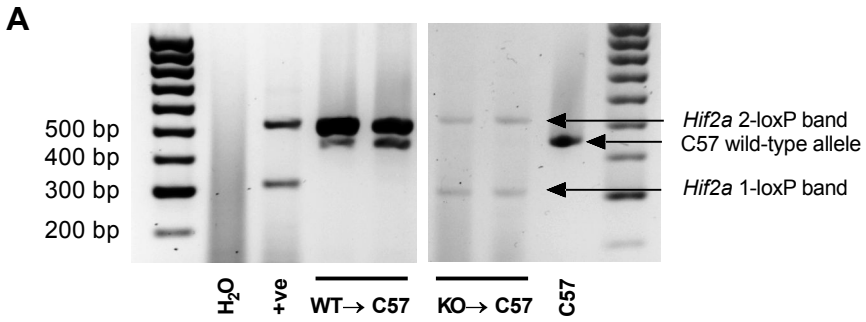

Supplemental figure 7