Supplementary Materials for

Hypoxia determines survival outcomes of bacterial infection through HIF-1alpha dependent re-programming of leukocyte metabolism

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This PDF file includes:

Other Supplementary Material for this manuscript includes the following:

Data source file (Microsoft Excel format) containing raw data for all the figures.

Materials and Methods

Skin lesion model

Wild-type C57BL/6 mice had the fur on their backs shaved with electric hair trimmers and were then with 50 µl of SH1000 at a concentration of 1 x 10^9 cfu/ml. Mice were weighed daily for 7 days and a photograph of the injected area was taken. On day 7 mice were sacrificed and the skin lesions dissected, weighed, snap frozen in liquid nitrogen and stored at -80˚C. ImageJ software (version 1.45, National Institutes of Health, USA) was used to trace the circumference of abnormal skin in each photographic image and skin lesion area was calculated.

Skin histology

Skin biopsies (6 mm diameter) of the injection site were placed in 10% buffered formalin prior to processing and staining with haematoxylin/eosin or rabbit polyclonal anti-MPO antibody (Abcam, Cambridge, UK).

Skin myeloperoxidase (MPO) activity

Skin biopsies were weighed then immediately frozen in liquid nitrogen before determining MPO activity. In brief, the skin was chopped finely, homogenised and placed in hexadecyltrimethylammonium bromide containing 50 mM potassium phosphate (HTAB buffer) at pH 6.0. Homogenisation was performed using either a glass pestle and mortar or with stainless steel beads in a Bullet Blender® (Next Advance, Averill Park, USA). Samples were sonicated in a Bioruptor™ iced water bath (Diagenode Europe SA, Liège, Belgium), then frozen and thawed 4 times before a further 5 minute period of sonication and subsequent centrifugation at 14000 g for 30 minutes at 4˚C. Supernatant was added to O-dianisidine solution and absorbance at a wavelength of 450 nm was read at 30 seconds and 90 seconds to give the relative myeloperoxidase activity.

Bacterial counts in homogenised skin

Skin was homogenised in PBS and numbers of colony forming units in skin homogenates were calculated using the Miles and Misra method and normalised per gram of skin.

Measurements in plasma and serum

Blood from mice injected with SH1000 or vehicle was harvested at 3, 6 or 12 hours and spun at 350g for 10 minutes to generate plasma. To analyse cytokines, cytometric bead arrays (CBA) using BD™ CBA flex sets (murine Il-1β, Il-6, Il-10, TNF-α, KC, MCP-1) were performed as per manufacturer's instructions.

Serum was generated by allowing blood to clot at room temperature before centrifugation at 500g for 10 minutes before being transported on ice to a veterinary laboratory (Nationwide Veterinary Laboratories, Leeds) and analysed for makers of liver (aspartate transaminase), kidney (creatinine) and pancreatic dysfunction (lipase).

MMP-9, lactate, ATP, PGE2, VEGF, catecholamines and corticosterone were measured by ELISA. Lipid peroxidation in plasma was determined using a MDA quantification kit (OxiSelect™, TBARS assay kit, Cell Biolabs Inc., San Diego, USA) as per manufacturer's instructions.

Nitric oxide measurements

Frozen plasma obtained from mice infected with *S. aureus* or injected with PBS was analysed for nitric oxide content. In brief, plasma was passed over a 10 kDa filter column and analysed on a Sievers Nitric Oxide Analyser NOA 280i (GE Water & Process Technologies, Boulder, USA) following manufacturer instructions. Total NO_x concentration was determined from a calibration curve constructed with readings obtained from serially diluted nitrate solutions.

Assessment of lung injury

Bronchoalveolar lavage was performed via cannulation of the trachea in anaesthetised mice, while in other animals the lungs were fixed by instillation of buffered formalin. In separate experiments lung wet:dry weight ratio was calculated.

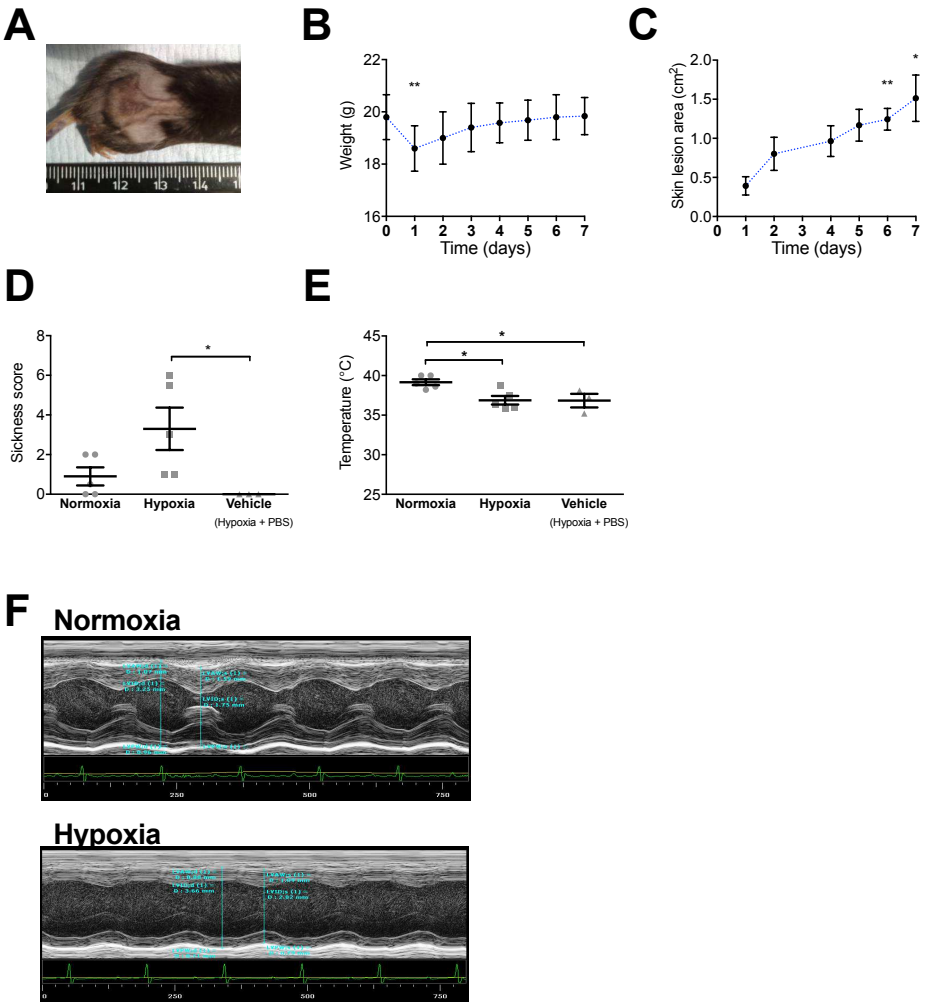

Fig. S1. Hypoxia induces hypothermia, sickness behaviour and cardiac dysfunction in mice infected subcutaneously with *Staphylococcus aureus*. (**A-C**) Representative photograph of a *S. aureus* skin lesion (**A**) on a mouse injected subcutaneously with $5x10^7$ cfu of SH1000 *S. aureus* and maintained in normoxia for 7 days. Daily mouse body weights (**B**) and skin lesion area (**C**) were measured. (**B**) ** P = 0.0015 vs. day 0; (**C**) ** P = 0.00576 vs. day 1; * P = 0.0223 vs. day 1; n = 5; repeated measures ANOVA with Dunnett's post test. (**D-F**) Sickness scores (**D**) and temperatures (**E**) of mice 6 hours after injection of *S. aureus* or vehicle in normoxia (21% O_2) or hypoxia (10% O_2), with representative echocardiographic short axis M mode view 12 hours after *S. aureus* injection (**F**). (**D**) * P = 0.0493 Hypoxia (n = 5) vs. Vehicle (n = 3); (**E**) * P = 0.0248 Hypoxia (n = 5) vs. Vehicle (n = 3); * P = 0.045 Normoxia (n = 5) vs. Vehicle (n = 3); one-way ANOVA with Tukey post tests. Points (**B-C**) or horizontal lines (**D-E**) are mean +/- SEM.

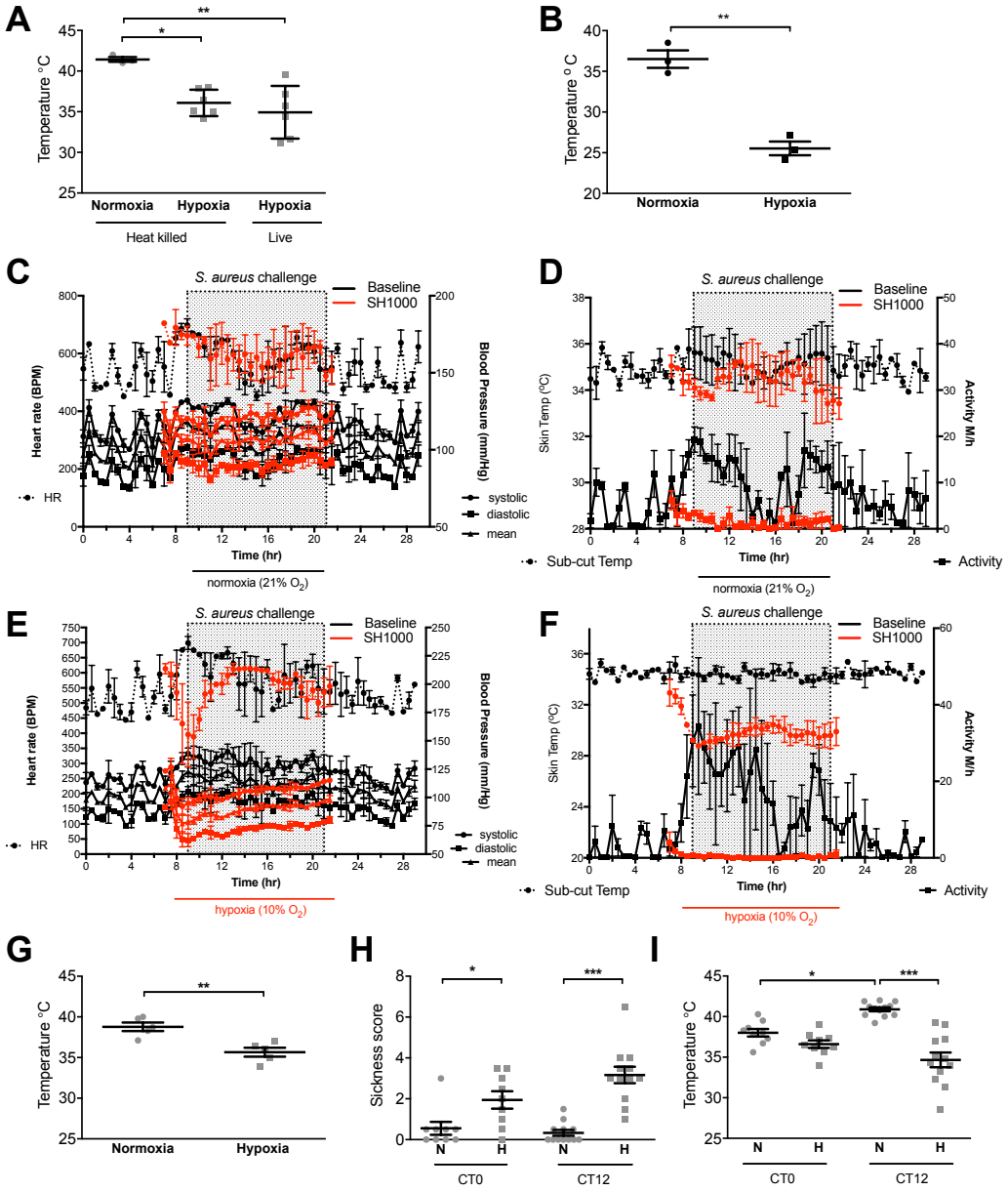

Fig S2. The phenotype observed in hypoxic infected C57BL/6 mice is reproduced by heat-killed SH1000, by intraperitoneal LPS, in an outbred mouse strain and following dawn or dusk infection.(**A**) Live (5x10^7 cfu) or heat-killed (5x10^8 cfu) SH1000 were injected subcutaneously and temperature recorded after 12 hours in normoxia or hypoxia. * P = 0.0186 Normoxia (n = 3) vs. Hypoxia heat killed (n = 6); ** P = 0.00539 Normoxia vs. Hypoxia live (n = 6); one-way ANOVA with Tukey's post test. (**B**) LPS was injected intraperitoneally and temperatures were recorded after 4 hours in normoxia or hypoxia. ** P = 0.00132; n = 3; unpaired t-test. (**C-F**) Heat-killed (5x10^8 cfu) SH1000 were injected subcutaneously into mice pre-implanted with radiotelemetery devices and heart rate (**C, E**), blood pressure (**C, E**), skin temperature (**D, F**) and activity (**D, F**) measured in real time in normoxia (**C, D**) and hypoxia (**E, F**) and plotted against paired baseline data in normoxia. Data are mean +/- SEM, n = 2 per condition. (**G**) MF1 mice were infected with 5x10^7 cfu SH1000 and temperature recorded after 12 hours in normoxia or hypoxia. ** P = 0.00344; n = 5; unpaired t-test. (**H, I**) C57BL/6 mice were injected with SH1000 at CT0 or CT12 and sickness score (**H**) and temperature (**I**) recorded after 12 hours. (**H**) * P = 0.047 CT0 N vs. CT0 H; n = 9; *** P = 0.000001 CT12 N vs. CT12 H; n = 12; two-way ANOVA with Tukey post test. (**I**) * P = 0.0103 CT0 N (n = 9) vs. CT12 N (n = 12); *** P = 0.000001 CT12 N vs. CT12 H; n = 12; two-way ANOVA with Tukey post test. Data are mean +/- SEM.

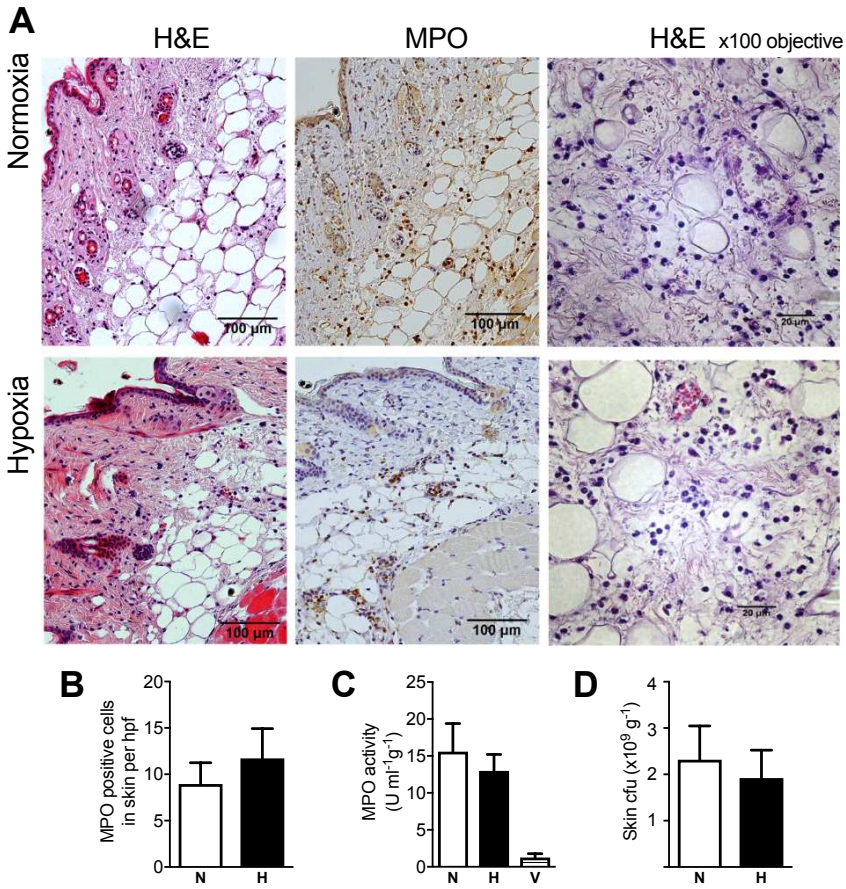

Fig. S3. Local immune responses are not impaired by hypoxia. Mice were injected with SH1000 and placed in normoxic (N, 21% O_2) or hypoxic (H, 10% O_2) conditions for 12 hours. (**A**) Representative photomicrographs of skin biopsy sections stained with haematoxylin and eosin (H&E) or anti-myeloperoxidase (MPO) antibody. (**B**) Counts of MPO positive cells per high powered field in skin tissue (n = 3). (**C**) Skin biopsies were homogenized in HTAB buffer. MPO activity was analysed and normalised per gram of skin tissue (n = 10 N, 10 H, 5 V). (**D**) Skin biopsies were weighed and homogenized in PBS before colony forming unit counts were obtained using the Miles and Misra method (n = 5 N, 6 H). Data are mean +/- SEM.

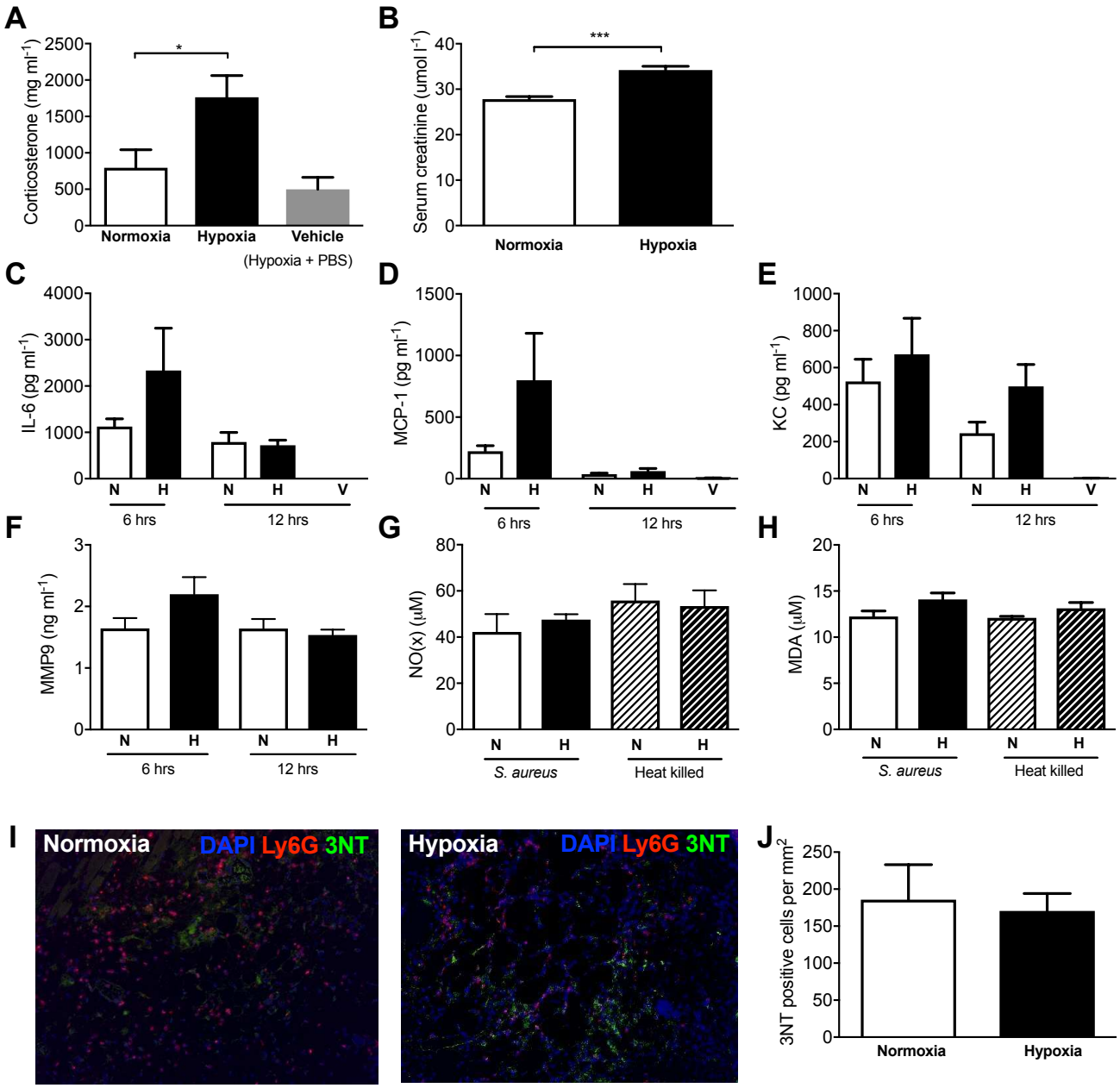

Fig. S4. Mice display stress responses to hypoxia but no demonstrable systemic inflammatory or oxidative response. Mice were injected with SH1000 and placed in normoxic (N, 21% O_2) or hypoxic (H, 10% O_2) conditions for 3, 6 or 12 hrs. Serum obtained at 12 hrs was analysed for (**A**) corticosterone and (**B**) creatinine. (**A**) * P = 0.0465 Normoxia vs. Hypoxia; n = 4; (**B**) *** P = 0.000271 Normoxia vs. Hypoxia; n = 5; unpaired t-tests. Plasma was analysed for (**C**) IL-6, (**D**) MCP-1, (**E**) KC, (**F**) MMP9, (**G**) NO_x, and (**H**) malondialdehyde (MDA) expression. (**I-J**) 12 hour skin biopsy sections were co-stained with DAPI, Ly6G and 3NT and 3NT positive cells determined per mm^2; n = 4. (**C-E**) minimum n = 5; (**F**) 6 hrs n = 3, 12 hrs n = 4; (**G**) minimum n = 3; (**H**) n = 5. Data are mean +/- SEM.

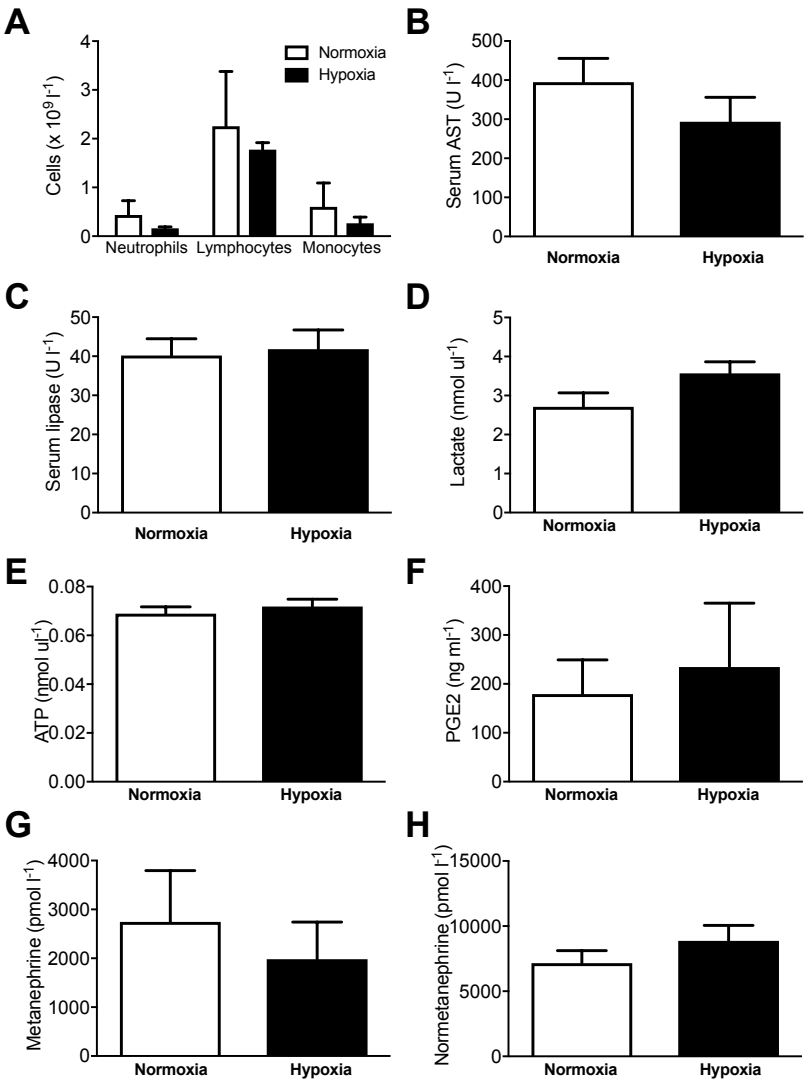

Fig. S5. Mice display preserved gross organ function in hypoxia. Mice were injected with SH1000 and placed in normoxic (21% O_2) or hypoxic (10% O_2) conditions for 6 to12 hrs. (**A**) At 6hrs total peripheral leukocyte counts were performed (n = 2). Serum obtained at 12 hrs was analysed for (**B**) aspartate transaminase (AST), (**C**) lipase, (**D**) prostaglandin E2 (PGE2), (**E**) lactate, (**F**) adenosine triphosphate (ATP), (**G**) metanephrine and (**H**) normetanephrine levels. Data are mean +/- SEM for a minimum n = 4.

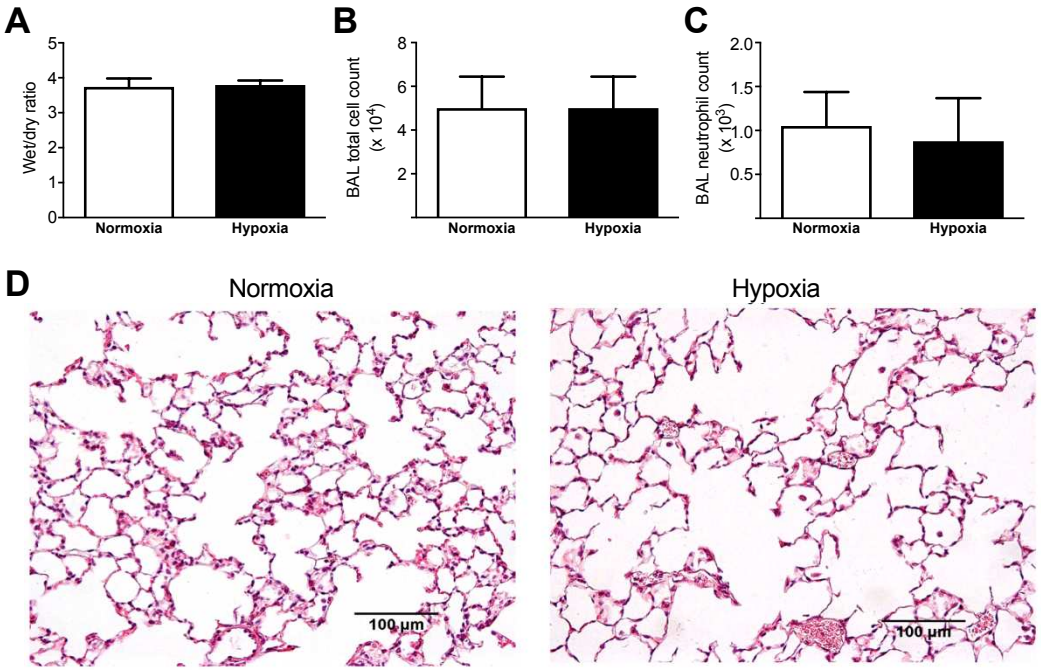

Fig. S6. No evidence of increased pulmonary oedema or pulmonary immune infiltration in hypoxic animals infected with *S. aureus.* Mice were placed in hypoxia or normoxia for 12 hours following infection with SH1000. Lung oedema was assessed by wet to dry lung weight ratio (**A**). Bronchoalveolar lavage was performed in anaesthetised animals and (**B**) total cell count and (**C**) neutrophil counts were calculated. Data are mean and SEM, n = 3. (**D**) Representative photographs of lung tissue sections from infected normoxic and hypoxic mice stained with haematoxylin and eosin. Original magnification x200.

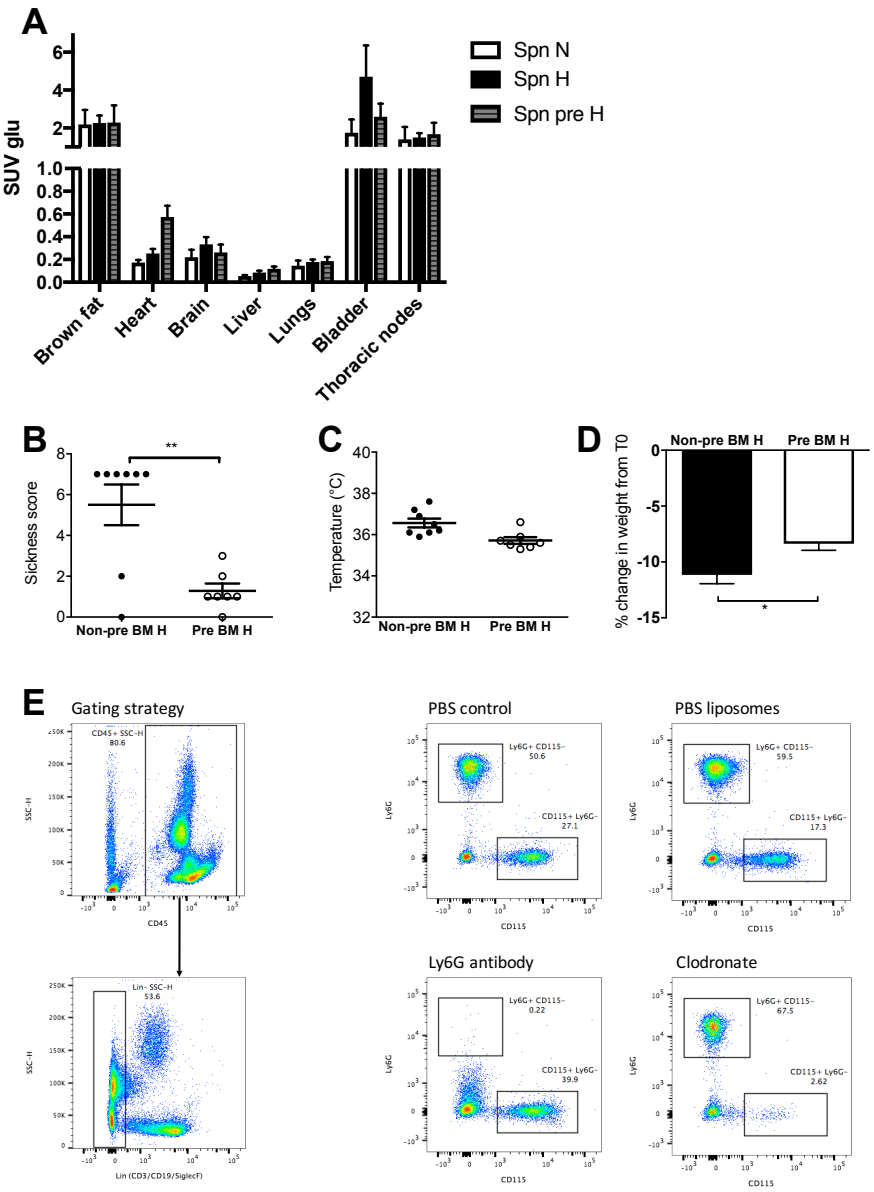

Fig. S7. Hypoxic preconditioning alters glucose uptake into tissues and protection is conveyed via the bone marrow. (**A**) Mice were challenged with intra-tracheal *S. pneumoniae* (Spn) and housed in normoxia (N), hypoxia (H) or in hypoxia following preconditioning (Pre H). ^{18}F-FDG was administered 23 hours following installation of *S. pneumoniae* and in vivo standardised glucose uptake values determined by positron emission tomography (PET); n = 3 N, 4 H, 3 Pre H. (**B-D**) Mice were acclimatised to hypoxia for 7 days, bone marrow harvested and injected into wildtype C57BL/6 mice pre-irradiated with 12 fractions of 1Gy (Pre BM), with non-acclimatised mice used as marrow donor controls (Non-pre BM). Following 3 weeks re-constitution mice were challenged with intra-tracheal *S. pneumoniae* in hypoxia and sickness scores (**B**), temperatures (**C**) and weights (**D**) recorded after 20 hours. Bars (**A, D**) or horizontal lines (**B, C**) are mean +/- SEM. (**B**) ** *P* = 0.0024, non-pre BM vs Pre BM; n = 8 N, 7 H; unpaired t-test. (**D**) * *P* = 0.0166, non-pre BM vs Pre BM; n = 8 N, 7 H; unpaired t-test. (**E**) Mice were injected intraperitoneally with PBS, anti-Ly6G, PBS containing liposomes or clodronate liposomes and 12 hrs later challenged with subcutaneous SH1000. After a further 12 hours, whole blood was collected, red cells lysed and cells stained using Ly6C FITC, Lineage (CD3, CD19), Siglec F PE, CD115 APC, 7-AAD, CD45 AF700, Ly6G Pacific Blue to deterrmine deletion efficiency. Flow plots are representative of n = 3.

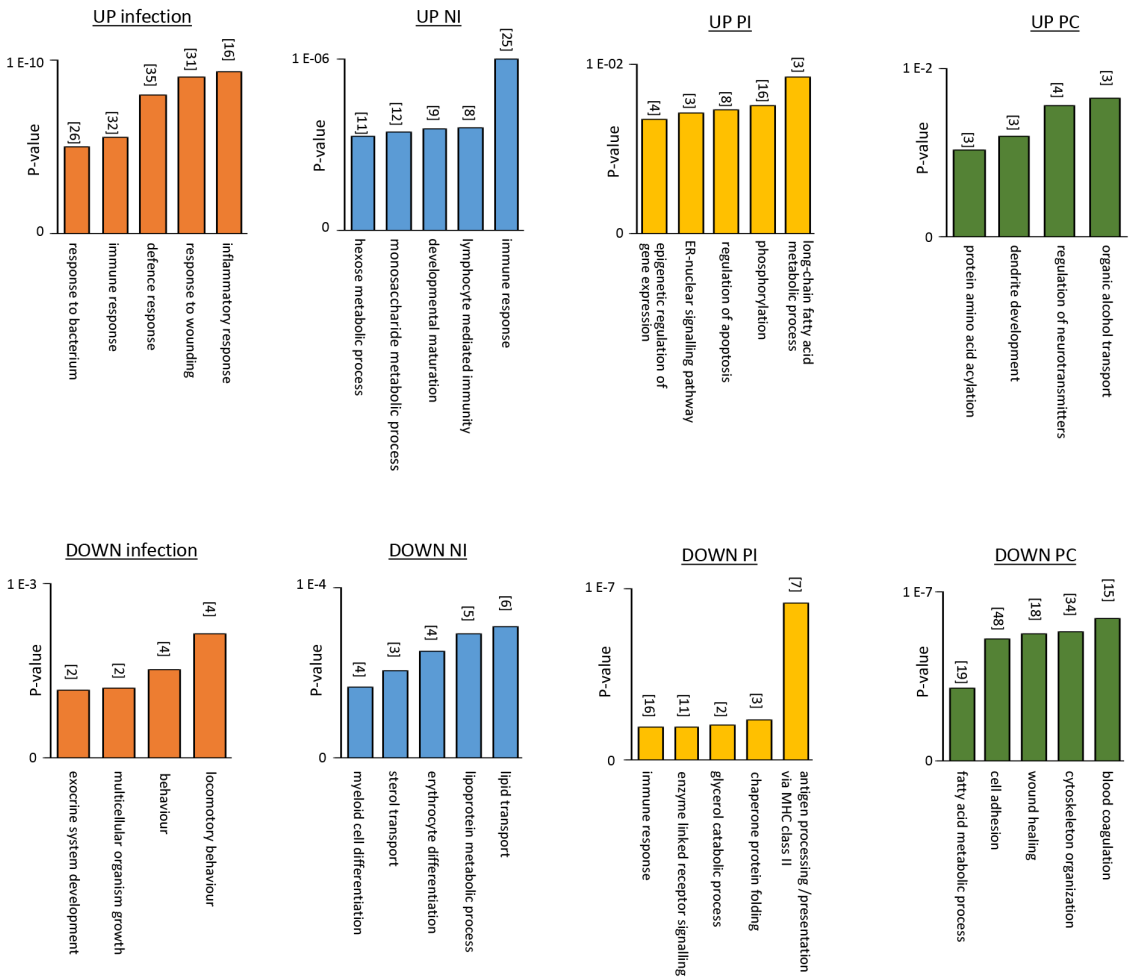

Fig. S8. Hypoxic preconditioning profoundly changes the global transcriptome of circulating leukocytes. Pathway analysis (GO Terms) was undertaken of strand specific gene expression patterns of blood leukocytes harvested from hypoxia naïve mice instilled with *S. pneumoniae* in 10% O_2 (naïve infected, NI) or vehicle control (naïve control, NC) or following hypoxic pre-conditioning (preconditioned infected, PI and preconditioned control, PC).

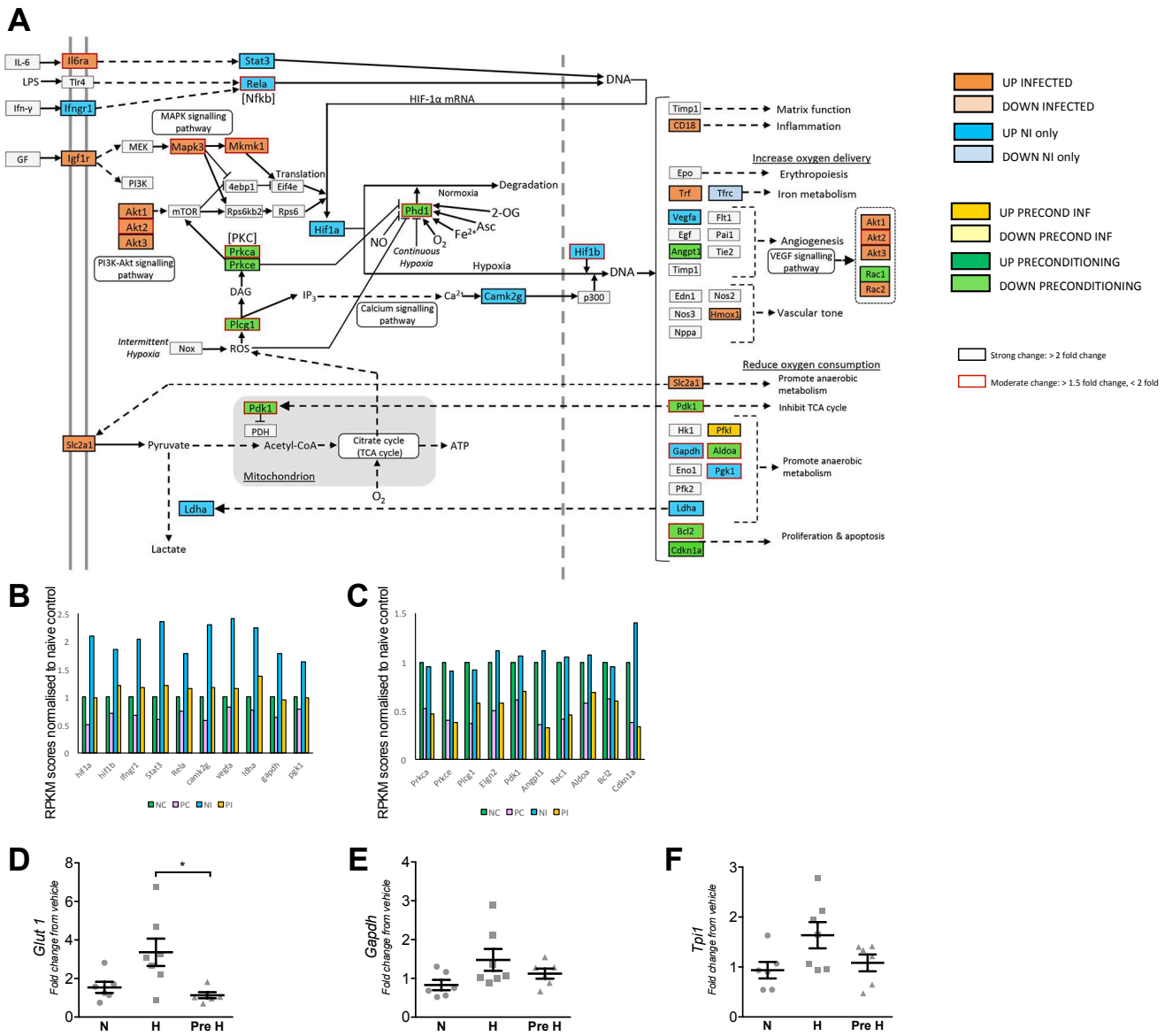

Fig. S9. Hypoxic preconditioning alters the HIF-1α pathway and down stream targets in circulating leukocytes. (**A-C**) Pathway analysis. KEGG based annotation pathway analysis was undertaken of strand specific gene expression patterns of blood leukocytes harvested from hypoxia naïve mice instilled with *S. pneumoniae* in 10% O_2 (naïve infected, NI) or vehicle control (naïve control, NC) or following hypoxic pre-conditioning (preconditioned infected, PI and preconditioned control, PC). Genes changing expression are color coded with strong changes (>2 FC) outlined by black boxes and moderate changes (>1.5 FC but <2 FC) outlined in red (**A**). Targeted HIF-1α signaling pathway analysis with genes induced by NI and suppressed with preconditioning (**B**), or globally suppressed by preconditioning (**C**). (**D-F**) RNA relative quantification. Leukocytes were harvested from mice injected with *S. pneumoniae* or vehicle in normoxia (N, 21% O_2), hypoxia (H, 10% O_2) or in hypoxia following preconditioning (Pre H), RNA extracted and expression of *Glut1*, *Gapdh* and *Tpi1* analyzed by real time PCR. Data were normalized to *Actb* and expression level in vehicle controls. Horizontal bars are mean +/- SEM. (**D**) * P = 0.0135, H vs Pre H; n = 6 N, 7 H, 6 Pre H; one-way ANOVA with Tukey's post-test.

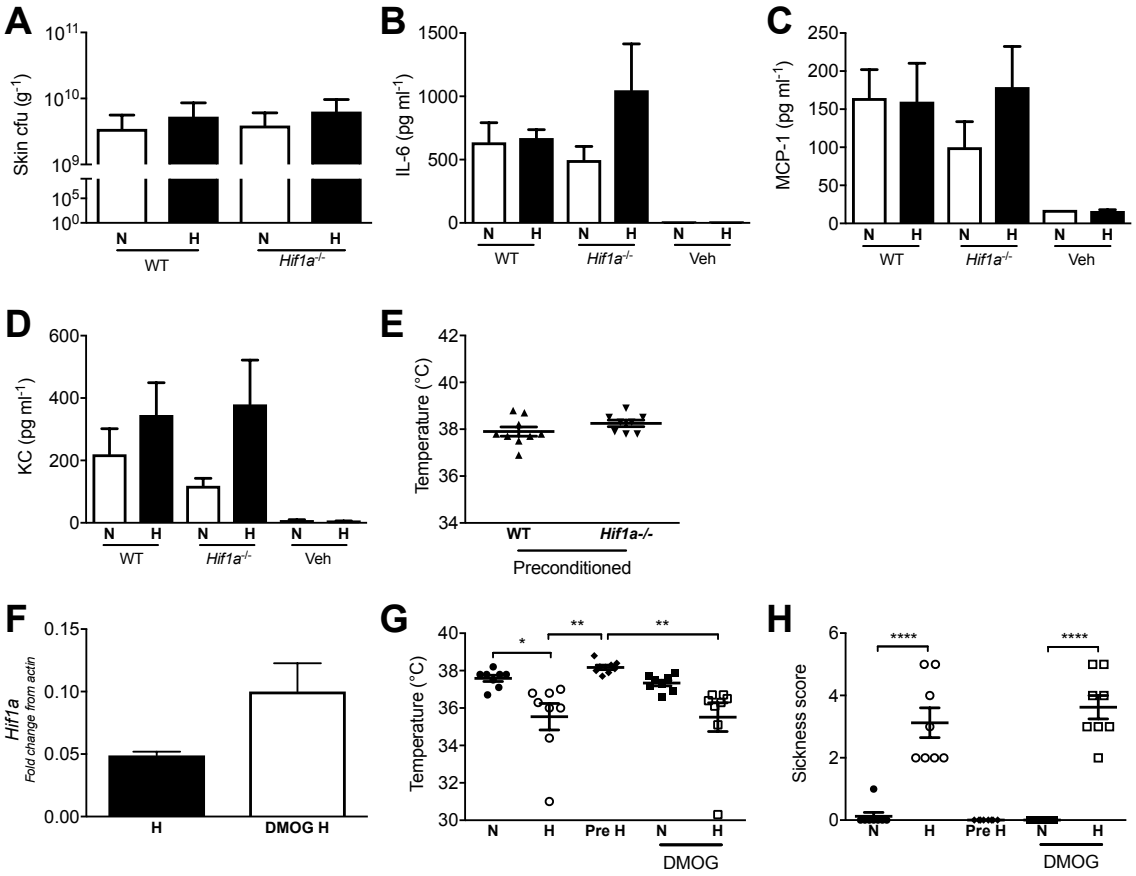

Fig. S10. Preserved bacterial load, cytokine responses and preconditioning responses with myeloid specific suppression of HIF-1α. (**A-D**) Acute hypoxia. Wild-type C57BL/6 (WT) and *Hif1a*$^{flox/flox}$;*LysMCre*$^{+/-}$ (*Hif1a*$^{-/-}$) mice were injected with SH1000 and placed in normoxic (N, 21% O_2) or hypoxic (H, 10% O_2) conditions for 12 hours. (**A**) Skin biopsies were weighed and homogenised in PBS before colony forming unit counts were obtained using the Miles Misra method. minimum n = 5. Plasma was analyzed for (**B**) IL-6, (**C**) MCP-1 and (**D**) KC expression; n = 3 N/H, 2 V; (**E**) Hypoxic preconditioning. Wild-type C57BL/6 (WT) and *Hif1a*$^{flox/flox}$;*LysMCre*$^{+/-}$ (*Hif1a*$^{-/-}$) mice were preconditioned in hypoxia (H, 10% O_2) for 7 days, challenged with concurrent subcutaneous SH1000 and hypoxia (H, 10% O_2) for 12 hours and rectal temperatures recorded; n = 9 WT, 9 *Hif1a*$^{-/-}$. (**F-H**) DMOG. Mice were exposed either to a pan hydroxylase inhibitor (DMOG) (8mg intraperitoneal DMOG alternate days) or hypoxia for 7 days (Pre H), challenged with subcutaneous SH1000 and housed in normoxia (N) or hypoxia (H). (**F**) Blood leukocytes were harvested for relative *Hif1a* RNA quantification by real time PCR following normalization to *Actb*; n = 2 H, 3 DMOG H. (**G**) Temperature and (**H**) sickness score were taken 12h after infection. Data are mean +/- SEM. (**G**) * P = 0.0361, N v.s H; ** P = 0.0038, H vs Pre H; ** P = 0.0035, Pre H vs. DMOG H; n = 8; (**H**) **** P <0.000001, N vs. H, **** P <0.000001 N DMOG vs. H DMOG; n = 8; one-way ANOVA with Tukey's post-tests.