

Supporting Information

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SI Materials and Methods

Mice. C57BL/6J mice were bred in house. B6.Rag1^{-/-} mice (B6.129S7-Rag1^{tm1Mom}/J), FVB.129S6-Gt(ROSA)26Sor^{tm2(HIF1A)luc}Kael/J mice (referred to as ODD-luciferase mice), Foxp3-GFP mice (B6.Cg-Foxp3^{tm2Tch}/J), and Ubc-GFP mice [C57BL/6-Tg(UBC-GFP)30Scha/J] were obtained from Jackson Laboratory. Hypoxia-inducible factor (Hif)1a-flox/flox × LckCre mice were the F₂ progeny of Hif1a-flox/flox mice (B6.129-Hif1a^{tm3Rsj}/J) and LckCre mice [B6.Cg-Tg(Lck-cre)548Jxm/J], with mice obtained from Jackson Laboratory and genotyped according to recommended protocols. All mouse experiments were done using age- and sex-matched mice, with mice typically used between 8 and 12 wk of age. All experiments were done in accordance with local and national guidelines and institutional approval.

T-Cell Purification. T cells were purified from the spleens of mice, mechanically disrupted over a 100- μ m filter, and subjected to magnetic bead enrichment for CD4 T cells, using a CD4 T-cell isolation kit (Miltenyi Biotec). Enriched CD4 T cells were then subjected to FACS purification on a FACSAria (BD), with naive CD4 T cells purified on the basis of the cell surface phenotype CD4⁺ CD62L^{high} CD44^{low}. Additionally, FoxP3-GFP mice were used to purify FoxP3-expressing cells for subsequent quantitative real-time reverse-transcriptase PCR (qPCR) analysis, with regulatory T cells (Tregs) identified as CD4⁺ CD25⁺ GFP⁺.

In Vitro T-Cell Cultures. Jurkat T cells were cultured in RPMI 1640 (Gibco) containing 10% FBS (i.e., 10 mL FBS per 100 mL media), L-glutamine, and penicillin/streptomycin and were routinely kept at a density between 1 and 10 × 10⁵ cells/mL. Bulk splenocytes or purified CD4 T cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Gibco) containing 5% FBS, L-glutamine, penicillin/streptomycin, and β -mercaptoethanol (50 μ M). All cultures were done using cells at a concentration of 1 × 10⁶ cells/mL. Unstimulated cultures were supplemented with 1–2 ng/mL of recombinant mouse IL-7 (eBioscience). For cultures subjected to T-cell receptor stimulation, either bulk splenocytes were stimulated with 1 μ g/mL soluble anti-CD3 ϵ (clone 145-2C11; eBioscience) or naive CD4 T cells were stimulated with anti-CD3/anti-CD28 microbeads (using a 1:3 ratio of beads to cells; Dynabeads Mouse T-Activator CD3/CD28; Invitrogen). Stimulated cultures were supplemented with 10 ng/mL of recombinant mouse IL-2 (eBioscience). In vitro Treg differentiation assays were done for 3 d. In certain assays, exogenous TGF- β was added to the culture, using recombinant human TGF- β 1 (eBioscience). In assays that tested the role of TGF- β , cells were treated with a neutralizing TGF- β antibody (1D11 at final concentration of 10 μ g/mL; R&D Systems). In vitro Treg suppression assays were performed in round-bottom 96-well plates, in which 5 × 10⁴ Violet Trace-labeled CD4⁺ CD25⁻ T cells were cocultured with irradiated CD90.2-depleted splenocytes and soluble anti-CD3 antibody (1 μ g/mL) and decreasing numbers of CD4⁺ CD25⁺ T cells (Tregs), using either control or Hif1 α -deficient T cells (1). T-cell populations were purified using a CD4⁺ CD25⁺ isolation kit (Miltenyi Biotec). Th17 differentiation cultures were done using bulk splenocytes cultured with soluble anti-CD3 (1 μ g/mL), rmIL-2 (10 ng/mL), rhTGF- β (5 ng/mL), rmIL-6 (10 ng/mL), anti-IFN- γ (10 μ g/mL; XMG1.2), and anti-IL-4 (10 μ g/mL; 11B11).

RNA Isolation and Real-Time PCR. Total RNA was extracted from cells or tissue by TRIzol, followed by cDNA synthesis using an

iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. qPCR (iCycler; Bio-Rad Laboratories) was performed to measure relative mRNA levels for various transcripts, with qPCR master mix containing 1 μ M sense and 1 μ M antisense primers with iQ SYBR Green (Bio-Rad Laboratories). For every assay, melt curve analysis was performed and samples with aberrant melt curves were discarded. All qPCR assays were standardized relative to β -actin levels. For hypoxic cultures, qPCR analysis routinely included measurement of PGK1, a known hypoxia-inducible gene, as a positive control.

Antibodies and Flow Cytometric Analysis. Antibodies were purchased from eBioscience, unless otherwise noted. Anti-mouse antibodies included CD4 (GK1.5), CD25 (PC61.5), CD44 (IM7), CD62L (MEL-14), c-Rel (1RELAH5), CTLA-4 (UC10-4B9), FoxP3 (FJK-16s), GITR (DTA-1), MHC class II (M5/114.15.2), and ROR γ (AFKJS-9). Anti-human FoxP3 antibody was clone PCH101. Single-cell suspensions of cultured cells or disrupted tissue were stained with a mixture of antibodies for 30 min at room temperature in the dark, in staining buffer containing an anti-Fc receptor antibody (2.4G2). When cells were stained for FoxP3, staining was done using FoxP3 staining buffer according to manufacturer's instructions (eBioscience). CTLA-4 stain was routinely done on permeabilized cells to measure total protein. Unless stated otherwise, all stains included a viability dye to identify viable cells (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit; Invitrogen), with Tregs routinely identified as lymphocytes (on the basis of forward- and side-scatter profiles) that were viable, MHC class II negative cells that expressed CD4 and FoxP3. Analysis of Th17 differentiation was done by restimulating cultured T cells with PMA, ionomycin, and monensin for 5 h. All flow cytometry was done on an LSRII (BD), with compensation done using FACSDiva software. All flow cytometry data are shown on a log₁₀ scale.

Luciferase Assays. Jurkat cells were transfected by Amaxa nucleofection with either the parent pGL4.17 plasmid (Promega) or pGL4.17 containing a 1,000-bp fragment containing sequence from 920 bp of proximal 5' sequences through position +176 relative to the transcription start site of the human FOXP3 gene. FOXP3 promoter sequence was synthesized by GenScript and cloned into the pGL4.17 plasmid by restriction enzyme digest. Transfected cells were cultured in either normoxia or hypoxia for 24 h posttransfection, at which time cells were harvested and analyzed for relative luciferase assay by measuring firefly luciferase activity, which was standardized to relative amount of protein contained within each individual sample. To examine the hypoxic and TCR inducibility of HIF-1 α luciferase, magnetically enriched CD4 T cells from ODD-luciferase mice (2) were cultured in either normoxia or hypoxia for 18 h, with some samples cultured with anti-CD3/CD28 microbeads, IL-2 \pm TGF- β (0.75 ng/mL). Cells were then harvested and assessed for firefly luciferase activity, with data presented as values standardized to total protein.

Lentiviral HIF-1 α Knockdown. Jurkat cells were subjected to lentiviral transduction by spinfection (800 × g for 30 min, with polybrene), using preformed lentiviral particles (MISSION shRNA Lentiviral Transduction; Sigma-Aldrich) with sequence targeting human HIF-1 α (TRC no. TRCN0000003810, clone ID NM_001530.x1048s1c1, sequence CCGGGTGATGAAAGAA-TTACCGAATCTCGAGATTCGGTAATTCTTTTCATCACTT-

TTT). Transductants were placed into puromycin-containing media within 3 d of transduction, to select from transduced cells, and passaged comparably to untransduced cells until culture in hypoxia.

In Vitro Hypoxia and HIF Stabilization in Vitro and in Vivo. Cells were subjected to hypoxia (1% O₂) in a humidified hypoxia chamber (Coy Laboratory Products). Prolyl-hydroxylase (PHD) inhibition in Jurkats was done using PHD inhibitor AKB-6899 (100 μM). For in vivo experiments, mice were injected by i.p. injection with AKB-4924 diluted in 40% cyclodextran, with a working solution of 5 mg/kg, every day for 6 d. Mice were killed within 24 h after the last injection. AKB compounds were the kind gift of Robert Shalwitz (Akebia Therapeutics).

Whole-Body Hypobaric Hypoxia. C57BL/6J mice were housed in conditions that replicated partial pressure at sea level ($P_B = 760$ mmHg, 21% O₂ available) for 2 d, and then mice were either left at the partial pressure of sea level or placed at hypobaric pressure mimicking 19,200 feet of altitude (~10% oxygen, an acute whole-body hypoxic exposure). Mice were then assessed for the abundance of Tregs in spleen, defined by flow cytometry.

Primers for qPCR Analysis. All primers used for qPCR are indicated below, with a 5'–3' orientation, and with m referring to mouse and h referring to human. mbeta-actin, Fwd-CTAGGCACCAGGGTGTGAT, Rev-TGCCAGATCTTCTCCATGTC; mFoxP3, Fwd-TCTCCAGGTTGCTCAAAGTC, Rev-GCAGAAGTTGCTGCTTTAGG; mHif1a, Fwd-TCTGGAAGGTATGTGGCATT, Rev-AGGGTGGCAGAACATTTAT; mHif2a, Fwd-GGACAGCAAGACTTTTCTGA, Rev-TTTGGTTCATGTTCTCCGAAT; mPdk1, Fwd-GCAGATTGTTTGGAAATGGTC, Rev-TGCTCACATGGCTGACTTTA; mAhr, Fwd-CAGGCGCTGAATGGCTTTGTGC, Rev-GCCATGGGCTTCGTCCACTCC; mGata3, Fwd-TGTGTGAAGTCCGGGGCAACC, Rev-GCTGCCAGACGCTTCGCTT; mIkzf4, Fwd-CTGGCCAGCAAGGTGATGAAATCC, Rev-GTGGGGTGGAAACGCTTGCGT; mRora, Fwd-GATGTCAGCGGATCGCTCGTGG, Rev-GGGCGCGACATTTACCCATCG; mSmad3, Fwd-AGCATGGACGCAGGTTCTCCAA, Rev-GTTCCACGGCTGCATTCGGT; mStat3, Fwd-ACCCCGAAGCCGACCCAGGTA, Rev-ATTGCTGCAGGTCGTTGGTGTAC; mStat4, Fwd-CCCAGCCGTGCGAAGTCTCAAG, Rev-R-CCGTTTGCACCGTCATTCAGC; mStat6, Fwd-CATCACCATTGCACACGTCATC, Rev-TGAGCGAATGGACAGGTCTTTG; mTbx21, Fwd-GATCACTACTAAGCAAGGACGGC, Rev-AGACCACATCCACAAACATCCTG; hACTB, Fwd-GGTGGCTTTTAGGATGGCAAG, Rev-ACTGGAACGGTGAAGGTGACAG; hHIF1A, Fwd-CTATGTAGTTGTGGAAGTTATGC, Rev-ACTAGGCAATTTTGCTAAGAATGC. Human FOXP3 and PGK1 QuantiTect primers were obtained from Qiagen.

Chromatin Immunoprecipitation (ChIP) Assays. Chromatin immunoprecipitation assays were performed with Jurkat cells (2×10^6 per assay), using standard protocols. Approximately 1×10^7 cells were exposed to air (21% O₂) or hypoxia (1% O₂) for 6 h. Cells were pelleted ($200 \times g$, 5 min), resuspended in PBS, and cross-linked in a solution of 1% formaldehyde with gentle shaking for 10 min at room temperature. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were then washed twice with cold PBS, resuspended in lysis buffer I (5 mM Pipes, pH 8.0, 85 mM KCl, 0.5% Nonidet P-40)

supplemented with HALT protease inhibitors (Pierce), and incubated on ice for 10 min. Crude nuclear lysate was collected by centrifugation at $1,000 \times g$, resuspended in lysis buffer II (50 mM Tris-HCl, pH 8.1, 1% SDS, 5 mM EDTA) with HALT protease inhibitor mixture, and incubated on ice for 10 min. Cell lysates were sonicated five times for 15 s each, using a Biologics Sonicator, model 150V/T. Chromatin was cleared by centrifugation ($14,000 \times g$ for 10 min at 4 °C), diluted 10-fold with dilution buffer (20 mM Tris, pH 8.1; 2 mM EDTA; and 1% Triton X-100), and aliquoted for immunoprecipitation to an equivalent of 2×10^6 cells per assay. Chromatin was precleared for 2 h with Protein G-Sepharose beads (GE Healthcare Life Sciences) and incubated at 4 °C overnight with beads alone (mock), rabbit IgG (control), or rabbit polyclonal antibody against HIF-1α (Novus Biologicals). HIF-promoter complexes were immunoprecipitated for 2 h, using Protein G-Sepharose beads, and washed. IP complexes were eluted (0.1 M NaHCO₃, 1% SDS) and incubated at 65 °C overnight to reverse cross-linking, and DNA was isolated by column purification (Qiagen). HIF binding to human FoxP3 genomic (promoter) DNA was quantified by conventional PCR, using primers FoxP3 set 1: forward 5'-AAA GAC CCC AAA GGC TGA GG-3' and reverse 5'-GAA TCC CTG GCT CCC AGA ATC-3'. Real-time quantitative PCR was performed on 2 μL of immunoprecipitated genomic DNA template, using Power SYBR (Applied Biosystems) in a total volume of 20 μL, using primers designed to amplify a 54-bp region proximal to the FoxP3 hypoxia response element (HRE) at -819: forward 5'-GCC AGG GAT TCT CCG ACT CT-3' and reverse 5'-GCC CAC TGT CAT CCC CTA AA-3'.

Induction of CD45RB^{high} Colitis. CD4⁺ CD45RB^{high} cells were isolated from Ubc-GFP mice and sorted on a FACS Aria, purifying CD4⁺ CD45RB^{high} (typically defined as the highest 50% of cells by CD45RB expression). B6.Rag1-deficient mice were adoptively transferred with $2\text{--}5 \times 10^5$ cells per mouse by i.p. injection. Tregs from either *Hif1a* flox/flox mice without cre or from *Hif1a* flox/flox LckCre mice were isolated by magnetic enrichment (Treg isolation kit; Miltenyi Biotec) or by sorting CD4⁺ CD25⁺ T cells on a FACS Aria, with isolated Tregs then cotransferred into recipients at a 1:4 ratio (Tregs:CD45RB^{high} cells). Mice were monitored for weight loss weekly, with mice harvested within 8 wk of the initial adoptive transfer. Upon necropsy colonic length was measured, and tissues were processed for histology.

Tissue Processing and Assessment of the Severity of Colitis. Colons (distal to cecum) were excised, placed in cold PBS, opened longitudinally and fixed in 10% buffered formalin, paraffin embedded, cut into 3- to 5-mm sections, and stained with hematoxylin/eosin. The severity of colitis was assessed independently by a board-certified pathologist (P.J.) blinded to the treatment groups.

Software and Statistical Analysis. Data analysis and plotting were done using Prism 4.0c (GraphPad Software). Flow cytometric data were analyzed using FlowJo (TreeStar), with data displayed as high-resolution zebra plots showing outliers, using log₁₀ scales. Statistical analyses were performed using Prism 4.0c, with unpaired *t* tests or one-way ANOVA and posttest Tukey's correction for all other analyses. Nucleotide sequence analysis was done using MacVector (version 10.6.0), with human *FoxP3* locus structure defined by the Ensembl database.

1. Tao R, et al. (2007) Deacetylase inhibition promotes the generation and function of regulatory T cells. *Nat Med* 13:1299–1307.

2. Safran M, et al. (2006) Mouse model for noninvasive imaging of HIF prolyl hydroxylase activity: Assessment of an oral agent that stimulates erythropoietin production. *Proc Natl Acad Sci USA* 103:105–110.

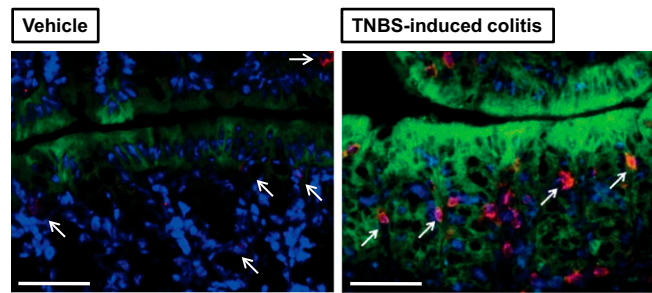


Fig. S1. TNBS (2,4,6-trinitrobenzenesulfonic acid)-induced colitis in mice is associated with an extension of hypoxia into the lamina propria and is colocalized with T-cell infiltrates. To study hypoxia as an environmental cue of the inflamed microenvironment, TNBS-induced colitis was used as model inflammatory disease. CD3⁺ T cells (red) infiltrate into areas of hypoxia (green) during colitis (Right) relative to normal colon tissue (Left). Shown is immunofluorescence on colonic tissue from either control mice (Left) or mice exposed to TNBS colitis for 4 d and stained with hypoxyprobe (Right) (nuclear counterstaining with DAPI, blue). White arrows indicate examples of CD3⁺ T cells in control or TNBS-treated mice. (Scale bars, 50 μ m.)

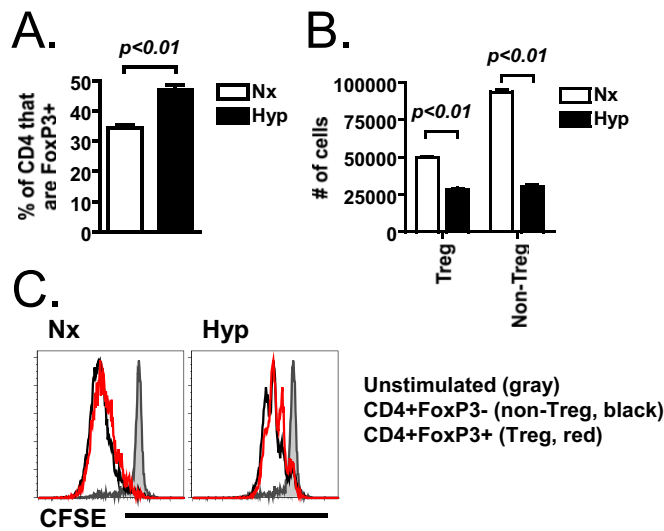


Fig. S2. Hypoxia enhances relative Treg abundance and is associated with reduced proliferation. (A) Hypoxia enhances the frequency of FoxP3⁺ CD4 T cells in bulk splenocyte culture, after *in vitro* stimulation with soluble anti-CD3 antibody (1 μ g/mL), IL-2, and TGF- β 1, comparing cells cultured in normoxia (Nx, white bar) or hypoxia (Hyp, black bar). (B) Hypoxia is associated with reduced cellularity relative to normoxia, when examining Tregs (CD4⁺FoxP3⁺) and non-Tregs (CD4⁺FoxP3⁻) at 3 d after culture. (C) Hypoxia is associated with reduced T-cell proliferation, in both CD4⁺FoxP3⁻ (non-Tregs, black line) and CD4⁺FoxP3⁺ (Tregs, red line) in hypoxia (Right) relative to normoxia (Left). Proliferation was measured by CFSE dilution, gating on lymphocytes that are viable, MHC class II negative, CD4⁺. Proliferation is shown in Nx (Left) or Hyp (Right) relative to total splenocytes that were CFSE labeled but left unstimulated (gray). Data are representative of two independent experiments, with three replicate cultures per experiment, and include statistical significance calculated by unpaired *t* test (comparing normoxia vs. hypoxia).

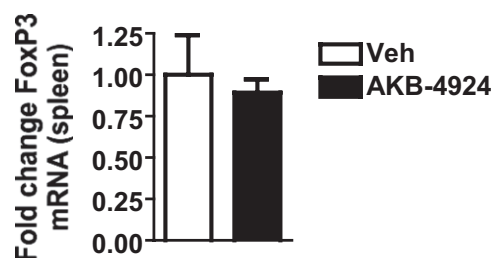


Fig. S3. Treatment of mice with a PHD inhibitor does not increase FoxP3 mRNA expression in the spleen. FoxP3 mRNA was measured by qPCR in spleen, in B6 mice treated with vehicle (control) or with AKB-4924. Data show mean \pm SEM, $n = 5$ mice per group, representative of two independent experiments.

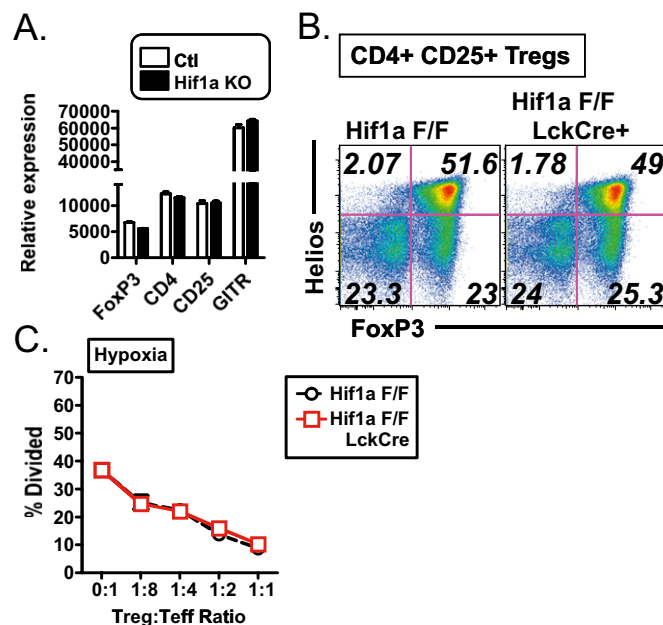


Fig. S4. *Hif1a*-deficient Treg protein expression profile and suppressive function under hypoxia. (A) *Hif1a*-deficient Tregs have comparable protein expression levels of multiple proteins, with data showing relative protein expression levels in FoxP3⁺ CD4 T cells from either control or *Hif1a* F/F LckCre⁺ splenocytes following a 3-d culture of bulk splenocytes with soluble anti-CD3 stimulation and IL-2. Data depict median fluorescent intensities from two independent experiments, in which three independent *Hif1a* flox/flox LckCre⁺ mice were tested. (B) Flow cytometric analysis of the CD4⁺CD25⁺ population of Tregs, isolated from a *Hif1a* floxed, control mouse (Left) or from a *Hif1a* floxed LckCre⁺, *Hif1a*-deficient mouse (Right). Data show events gated on viable lymphocytes, MHC class II negative, CD4⁺ cells, with data showing comparable Helios (y axis) and FoxP3 (x axis). Results are representative of two independent experiments. (C) In vitro Treg suppression assay using different ratios of CD4⁺ CD25⁺ T cells from *Hif1a* flox/flox (F/F) or *Hif1a* flox/flox LckCre⁺ mice cocultured with Violet Trace-labeled CD4⁺ CD25⁻ T cells from *Hif1a* flox/flox mice, irradiated APCs, and soluble anti-CD3 (1 μg/mL) under hypoxic conditions (1% O₂). T-cell proliferation was assessed 4 d poststimulation, with data obtained from four independent cultures, with cells isolated from three independent mice. Data depict the percentage of CD4⁺ CD25⁻ T cells that have undergone at least one round of division, showing mean ± SEM for four independent cultures, as measured by Violet Trace dilution at multiple different ratios of effector T cells to regulatory T cells. There were no statistically significant differences under these hypoxic conditions, in contrast to a deficit of *Hif1a*-deficient Tregs when cultured in normoxia (Fig. 7).

Table S1. Ct values and average fold change for Foxp3 mRNA in enriched CD4⁺ T cells cultured in either normoxia or hypoxia for 8 h

Experiment	Ct values								Average fold change
	Actin normoxia		Actin hypoxia		FoxP3 normoxia		FoxP3 hypoxia		
	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	Rep 1	Rep 2	
Experiment 1	25.98	25.12	25.35	26.29	33.94		28.26	30.30	23.12
Experiment 2	17.29	16.59	17.74	16.82	30.09	30.26	29.38	29.77	2.53
Experiment 3.1	21.60	21.52	22.80	22.85	28.78	29.09	28.51	28.92	7.26
Experiment 3.2	23.51	23.88	23.50	23.78	30.45	30.40	28.56	29.15	9.79

These data correspond to data presented in Fig. 1B, with each experiment composed of an independent preparation of MACS-enriched CD4 T cells, followed by 8 h of either normoxic or hypoxic exposure, followed by mRNA quantitation by qPCR. Rep, replicate.